THE ROLES OF ENDOGENOUS DAMAGE-ASSOCIATED MOLECULAR PATTERN (DAMP) PROTEINS IN COLORECTAL CANCER

Thesis presented in accordance with the requirements of the University of Liverpool for the degree of Doctor in Medicine (MD)

By

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March 2011

DECLARATION & STATEMENT OF ORIGINALITY

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The research was carried out in the Division of Surgery and Oncology, School of Cancer Studies, University of Liverpool, between November 2007 and January 2010.

ABSTRACT

Introduction: The endogenous Damage-associated Molecular Pattern (DAMP) molecules which play a vital role in chronic inflammatory process, have been implicated at stages of cancer initiation, proliferation and progression. Previous research in our unit has examined the expression of the DAMPs S100A8/A9 and HSP27 in colorectal tumours. The tumour microenvironment of colorectal tumours was found to contain fewer S100A8- but not S100A9-positive inflammatory cells, when the tumours were Smad4-negative, whilst HSP27 was shown to be a prognostic marker of poor outcome in rectal cancer patients. The current study explored the impact of S100A8, S100A9 and HSP27 on cancer cell motility and proliferation. To address the impact of environmental S100A8/A9 chemokines on tumour cells, the effects of exogenously added S100A8 and S100A9 proteins on cellular migration and proliferation in relation to Smad4 status of cancer cells were investigated. In addition, the potential of HSP27 expression to be a predictive marker of response to adjuvant therapy in colorectal cancer patients was examined, followed by *in vitro* assessment of the role of HSP27 in cell sensitivity to chemo-radiation.

Methods: Colorectal cancer cell lines were used to examine cell motility and proliferation with Modified Boyden Chamber and MTS assays respectively. Transient Smad4 and HSP27 depletions were performed using siRNAs. Recombinant GST-tagged S100A8, S100A9 and control GST proteins were generated and purified from *E. coli*. Previously characterised tumoural HSP27 expression in resected colorectal cancer specimens were correlated with 5-year cancer-specific survival of patients treated with adjuvant 5-fluorouracil (5-FU).

Following HSP27 depletion, changes to cell cycle profile were assessed using flow cytometry, and changes to cell sensitivity to chemotherapy (5-FU, irinotecan and oxaliplatin) and gamma irradiation were measured using annexin-V/propidium iodide apoptosis assays.

Results: Exogenously added S100A8 and S100A9 enhanced migration and proliferation in Smad4-positive and Smad4-negative cancer cells. However, transient depletion of Smad4 resulted in loss of responsiveness to exogenous S100A8, but not S100A9. S100A8 and S100A9 activated Smad4 signalling as evidenced by phosphorylation of Smad2/3; blockade of the receptor for the advanced glycation end products (RAGE) inhibited this response. Among patients treated with combined surgery and adjuvant 5-FU, patients with high tumoural HSP27 had poorer survival (P=0.01) compared to patients with low HSP27 and showed no improvement in survival compared to patients treated with surgery only (P=0.45). HSP27 depletion resulted in a significant reduction in cell motility, inhibited cell proliferation and led to cell cycle arrest, and significant increases in cell apoptosis in response to 5-FU, irinotecan, oxaliplatin and gamma radiation.

Conclusion: This study provides evidence of the cancer-promoting activities of S100A8, S100A9 and HSP27, suggesting crucial roles of inflammatory mediators in carcinogenesis. Further research in modulating the immune response with respect to these DAMPs may provide attractive therapeutic targets for colorectal cancer treatment.

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge all those who have given invaluable guidance, advice, critique and support in completing this research. First and foremost, I would like to acknowledge the contribution of my supervisors Dr Eithne Costello, Reader in Molecular Biology, University of Liverpool, and Mr Paul Rooney, Consultant Surgeon in General and Colorectal Surgery, Royal Liverpool University Hospital NHS Trust. I would like to thank Dr Costello and Mr Rooney for their dynamic guidance, advice, and enthusiastic monitoring of the progress of the research. I am grateful for both of their help in making this research possible and an enjoyable experience. I appreciate the support, input and interest taken by Professor J P Neoptolemos, Head of the Division of Surgery and Oncology.

I would also like to thank everyone in the Division of Surgery and Oncology for the help rendered during my research. In particular, the post-doctoral research assistants Dr Sarah Tonack, Dr Taoufik Nedjadi and Dr Mark Aspinall O'Dea and postgraduate research students Ms Elizabeth Tweedle and Mr Adnan Sheikh who have been extremely patience in teaching me laboratory techniques.

Special thanks are to Division of Immunology, Division of Pathology and Division of Pharmacology, University of Liverpool for allowing and helping me in using their research equipment.

Finally I would like to thank my family and friends for their encouragement and support in the pursuit of my education, career and dreams.

PRIZES/AWARDS, PRESENTATIONS & PUBLICATIONS

Prizes/Awards

1. ***Poster with Commendation*** entitled 'Tumour heat shock protein (HSP)-27 expression as predictive factor of patient response to adjuvant 5-fluorouracil in Dukes C colorectal cancer' at the Annual Meeting of the Association of Surgeons of Great Britain and Ireland (ASGBI), 15/4/10.

2. ***Travel Scholarship*** entitled 'cross-talk between cancer cells and their surrounding host cells: the role of exogenous S100A8 and S100A9 and their relations to tumoural Smad4 status in carcinogenesis' at the 41st meeting of the European Pancreatic Club, Szeged, Hungary, 1-3/7/09.

3. ***Best Poster Presenter*** entitled 'cross-talk between cancer cells and their surrounding host cells: the role of S100A8 and S100A9 and their relations to Smad4 in carcinogenesis' at the Annual Meeting of the Liverpool and North West Society of Surgeons, The Liverpool Maritime Museum, 05/12/2008.

Presentations

International Conferences:

1. ***Poster presentation*** entitled 'S100A8 and S100A9 activate Smad signalling pathway via RAGE in pancreatic cancer cells' at the 42nd meeting of the European Pancreatic Club, Stockholm, Sweden, 16-10/6/2010.

2. ***Poster presentation*** entitled 'Loss of Smad4 expression is predictive of poor survival in node-negative but not in node-positive colorectal cancer' at the European Society of Coloproctology (ESCP), Prague, 23-26/09/2009.

3. ***Poster presentation*** entitled 'The role of exogenous S100A8 and S100A9 proteins and their relations to tumoural Smad4 status in colorectal carcinogenesis' at the European Society of Coloproctology (ESCP), Prague, 23-26/09/2009.

4. ***Oral presentation*** entitled 'cross-talk between cancer cells and their surrounding host cells: the role of exogenous S100A8 and S100A9 and their relations to tumoural Smad4 status in carcinogenesis' at the 41st meeting of the European Pancreatic Club, Szeged, Hungary, 1-3/7/2009.

5. ***Poster presentation*** entitled 'S100A8 and S100A9 increase pancreatic and colorectal cancer cell motility and proliferation' at the American Pancreatic Association, Chicago, US, 7-8/11/2008.

National Conferences:

1. ***Oral presentation*** entitled 'Tumour Heat Shock-27 expression as predictive factor of patient response to adjuvant 5-Fluorouracil in colorectal cancer' at the Association of Coloproctology of Great Britain and Ireland (ACPGBI) Annual Meeting, Bournemouth, 28-30/06/2010.

2. ***Poster presentation*** entitled 'Tumour Heat Shock-27 expression as predictive factor of patient response to adjuvant 5-Fluorouracil in colorectal cancer' at the Association of Surgeons of Great Britain and Ireland (ASGBI), Liverpool Convention Centre, 15/4/10.

3. ***Poster presentation*** entitled 'S100A8 and S100A9 increase pancreatic and colorectal cancer cell motility and proliferation' at the National Cancer Research Institute Cancer Conference, The International Convention Centre, Birmingham, 5-8/10/2008.

Regional/Local Meetings:

1. ***Oral presentation*** entitled 'Tumour Heat Shock-27 expression as predictive factor of patient response to adjuvant 5-Fluorouracil in colorectal cancer' at the Liverpool and North West Society of Surgeons, The Liverpool Maritime Museum, 04/12/2009.

2. ***Poster presentation*** entitled 'cross-talk between cancer cells and their surrounding host cells: the role of S100A8 and S100A9 and their relations to Smad4 in carcinogenesis' at the Liverpool and North West Society of Surgeons, The Liverpool Maritime Museum, 05/12/2008.

Publications

Original papers

1. **Ang CW**, Sheikh AA, Tweedle EM, Tonack S, Jenkins RE, Park KB, Nedjadi T, Khattak I, Azadeh B, Dodson A, Kalirai H, Schwarte-Waldhoff I, Neoptolemos JP, Rooney PS, Costello E. Loss of Smad4 expression is associated with fewer S100A8-positive stromal monocytes in colorectal cancer and attenuated response to S100A8 but not S100A9 in colorectal and pancreatic cells. *Carcinogenesis* 2010; 31 (9): 1541-1551. PMID: 20622003.

2. Tweedle EM, Khattak I, **Ang CW**, Nedjadi T, Jenkins RE, Park BK, Kalirai H, Dodson A, Azadeh B, Terlizzo M, Greenhalf W, Neoptolemos JP, Rooney PS, Costello E. Low Molecular Weight Heat Shock Protein, HSP27 is a prognostic indicator in rectal cancer, but not colon cancer. *GUT* 2010; 59 (11): 1501-1510. PMID: 20947885.

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1. **Ang CW**, Altaf K, Tweedle E, Khattak I, Azadeh B, Terlizzo M, Myint S, Neoptolemos JP, Rooney PS, Costello E. Tumour Heat Shock-27 expression as predictive factor of patient response to adjuvant 5-Fluorouracil in colorectal cancer. *Colorectal Disease* 2010; 12 (S1): 6.

2. **Ang CW**, Tweedle EM, Neoptolemos JP, Rooney PS, Costello E. Tumour heat shock protein (HSP)-27 expression as predictive factor of patient response to adjuvant 5-fluorouracil in Dukes C colorectal cancer. *British Journal of Surgery* 2010; 97 (S2): 83.

3. **Ang CW**, Sheikh A, Tweedle E, Tonack S, Schwarte-Waldhoff I, Neoptolemos J, Rooney P, Costello E. The role of exogenous S100A8 and S100A9 proteins and their relations to tumoural Smad4 status in colorectal carcinogenesis. *Colorectal Disease* 2009; 11 (S2): 41.

4. **Ang CW**, Tweedle E, Azadeh B, Dodson A, Kalirai H, Neoptolemos J, Rooney P, Costello E. Loss of Smad4 expression is predictive of poor survival in node-negative but not node-positive colorectal cancer. *Colorectal Disease* 2009; 11 (S2): 41.

5. **Ang CW**, Sheikh AA, Tweedle E, Tonack S, Schwarte-Waldhoff I, Rooney P, Neoptolemos J, Costello E. 'Cross-talk' between cancer cells and their surrounding host cells: the role of exogenous S100A8 and S100A9 and their relations to tumoural Smad4 status in carcinogenesis. *Pancreatology* 2009; 9: 445.

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8. Sheikh AA, **Ang CW**, Tonack S, Tweedle E, Schwarte-Waldhoff I, Neoptolemos J, Costello E. S100A8 and S100A9 increase pancreatic and colorectal cancer cell motility and proliferation. *Pancreas* 2008; 37 (4): 496.

9. Sheikh AA, Tonack S, **Ang CW**, Nedjadi T, Dodson A, Campbell F, Neoptolemos JP, Costello E. Analysis of S100A8+, S100A9+ and CD68+ tumour-associated inflammatory cells in pancreatic cancer. *British Journal of Surgery* 2008; 95 (S7): 23.

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LIST OF ABBREVIATIONS

2-DE	Two-dimensional electrophoresis
5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
APC	Adenomatous polyposis coli
ASO	Antisense oligonucleotide
BAX	B cell lymphoma-2-associated X protein
BCA	Bicinchoninic acid
BMPR	Bone morphogenetic protein receptor
BRAF	B-Raf proto-oncogene serine/threonine-protein kinase
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal-instability pathway
CpG	Cytosine phosphoguanosine
CRC	Colorectal cancer
CRI	Cancer-related inflammation
CS	Cigarette smoking
DAMPs	Damage-associated molecular pattern proteins
DCC	Deleted in colorectal cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECL	Enhanced Chemo-Luminescense
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EGTM	European Group on Tumours Markers
FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
FOBT	Faecal occult blood test
FOLFOX	5-FU, leucovorin and oxaliplatin combination regimen
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GST	Glutathione S-transferase
Gy	Gray
IBD	Inflammatory bowel disease
IGF-R	Insulin-like growth factor II receptor
HNPCC	Hereditary non-polyposis colorectal cancer
HSF	Heat shock factor
HSP	Heat shock protein
IC_{50}	50% inhibitory concentration
IFL	Irinotecan, 5-FU and leucovorin combination regimen
kDa	Kilo Dalton
LC-MS/MS	Liquid-chromatography tandem mass spectrometry
LOH	Loss of heterozygosity
LV	Leucovorin
MAPK	Mitogen activated protein kinase
MAPKAPK	Mitogen activated protein kinase activated protein kinase

MDSCs	Myeloid-derived suppressor cells
MLH	Mutl homologue
MMR	Mismatch repair
MMS	Microsatellite stable tumours
MS	Mass spectrometry
MSH	MutS homolog
MSI	Microsatellite-instability pathway
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NSAIDs	Non-steroidal anti-inflammatory drugs
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PMS	Post-meiotic segregation increased
PTEN	Phosphatase and tensin homolog
QUASAR	Quick and Simple and Reliable Study
RAGE	Receptor for advanced glycation end products
SAPK	Stress-activated protein kinase
SDS	Sodium Dodecyl Sulfate
siRNA	Short interfering RNA
Smad	Mothers against decapentaplegic homologue (Drosophila)
TBST	Tris buffer saline Tween
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNM	Tumour, node, metastases
UICC	International Union Against Cancer
UK	United Kingdom

CHAPTER 1:
INTRODUCTION

1.1 Colorectal cancer (CRC)

CRC is the third most commonly diagnosed cancer in the UK after breast and lung. In 2007, over thirty eight thousand new cases of large bowel cancer were registered in the UK, of which around two-thirds occurred in the colon and one-third in the rectum[1]. This equates to around 100 new cases of CRC diagnosed each day, in which 54 % are in male patients. According to the National Bowel Cancer Audit Project 2007[2], 28 % of CRC patients are of working age (< 65), 12 % over 85 and 4 % over 95 (median age 73 years).

Most cases of CRC arise in the left side of the large intestine, with the sigmoid colon and rectum being the most prevalent sites. Adenocarcinoma (mucinous or signet ring types) is the commonest type of CRC. Other cellular types include scirrhous, neuroendocrine and carcinoid tumours. It has been reported that synchronous tumours occur in 4-5 % of cases[3]. CRC can spread directly to the surrounding structures, or via the lymphatic, blood and transcoelomic routes.

CRC is the second most common cause of death from cancer in the UK after lung cancer. The majority of deaths occurred in elderly patients, 80 % in patients aged 65 and over and almost two-fifths in the over 80s. In contrast to incidence trends, bowel cancer death rates have been falling continuously since the early 1990s and over the last decade death rates have dropped by around 13 % attributed to medical advances[1].

1.1.1 Surgical anatomy of the colon and rectum

The large intestine, consisting of the colon and rectum, extends from ileocaecal valve to the anus. It measures approximately 1.5 m long and 4 cm in diameter. The colon has the following subdivisions: caecum, ascending colon, transverse colon, descending colon and sigmoid colon[4]. In clinical practice, cancers that occur in these subdivisions are collectively known as colonic cancers.

In the pelvis, at the level of the second to third sacral vertebra, the sigmoid colon joins the rectum. The rectum is defined as the distal segment of the large bowel lying below the peritoneal reflection. On rigid sigmoidoscopy, it extends for approximately 15 cm from the anal verge. In surgical oncology practice, the anal verge has been used as the anatomical landmark for benchmarking rectal tumours as it affects permanent colostomy rates, the percentage of anastomotic leaks and local recurrence rates. It is the distance between the lower edge of the tumour and the anal verge that influences the type of neo-adjuvant or adjuvant therapy, the type of surgery and most importantly the outcome[5].

The epithelial mucosa lining the colon and rectum forms the innermost layer of the large intestine, followed by a thin sheet of muscle called the muscularis mucosa. This is then surrounded by a submucosal layer of loose stromal tissue, followed by muscularis propria and subserosa/serosa[6]. It is the understanding of this microscopic anatomy that allows the pathologist to stage CRC following surgical resection.

The colon and rectum mucosa are simple columnar epithelia. Most food is absorbed before reaching the large intestine and hence there are no plicae

circulares, villi or cells that secrete digestive enzymes. However, the mucosa in the large intestine is thicker, its abundant crypts are deeper, and there are huge numbers of goblet cells in the crypts. This reflects the function of the large intestine. Lubricating mucus produced by goblets cells eases the passage of faeces and protects the intestinal wall from irritating acids and gases released by resident bacteria.

1.1.2 Aetiology and risk factors

1.1.2.1 Genetic basis of colorectal cancer, adenomatous polyps and hereditary CRC

Studies have shown that the accumulation of genetic mutations or alterations within epithelial cells of the colon and rectum result in colorectal cancer formation. Mutations in the adenomatous polyposis coli (APC) gene, genes on chromosome 18q, the *KRAS* and p53 genes may each represent alternative, multiple mutational pathways in colorectal carcinogenesis, with distinct clinical outcomes[7, 8].

Most CRCs are sporadic in occurrence, but 5 % occur as a result of hereditary genetic mutations. In hereditary CRC, the germline mutations in the tumour suppressor gene APC (in Familial Adenomatous Polyposis, FAP) and the DNA-mismatch repair genes (in Hereditary Non-Polypoidal Colorectal Cancer, HNPCC, also called Lynch syndrome) increase the lifetime risk of developing CRC to 80 - 100 % compared to the general population[9].

In approximately 85 % of sporadic CRCs, allelic losses, chromosomal amplifications and translocations have been identified[10]. Deletion at 1p and 8p,

as well as loss of heterozygosity (LOH) of 17p and 18q are frequent in CRC. Such alterations are characteristics of the chromosomal-instability pathway (CIN) with stability of microsatellite DNA – these are referred to as microsatellite stable (MMS) tumours (Figure 1)[11]. The second pathway which represents approximately 15 % of sporadic CRCs is known as the microsatellite-instability pathway (MSI). Such tumours display frameshift mutations and base-pair substitutions that are commonly found in short, tandemly repeated nucleotide sequences known as microsatellites. This population of CRCs is associated with mutations in DNA mismatch repair genes, which lead to an accumulation of widespread mutations in genes, such as the genes encoding type II TGF-β receptor, the apoptosis regulator BAX, the insulin-like growth factor II receptor and others[12, 13]. Lastly, epigenetic silencing through the cytosine phosphoguanosine (CpG) Island Methylator Phenotype (CIMP) has been proposed as a third pathway in CRCs, characterised by hypermethylation and functional silencing of CpG islands in various tumour-suppressor genes. The expression of these tumour-suppressor genes in cancer cells can be reduced or eliminated through 'silencing', a process with changes in methylation (the promoters of genes that are hypermethylated will be 'silenced') as an alternative mechanism to genetic mutation[14].

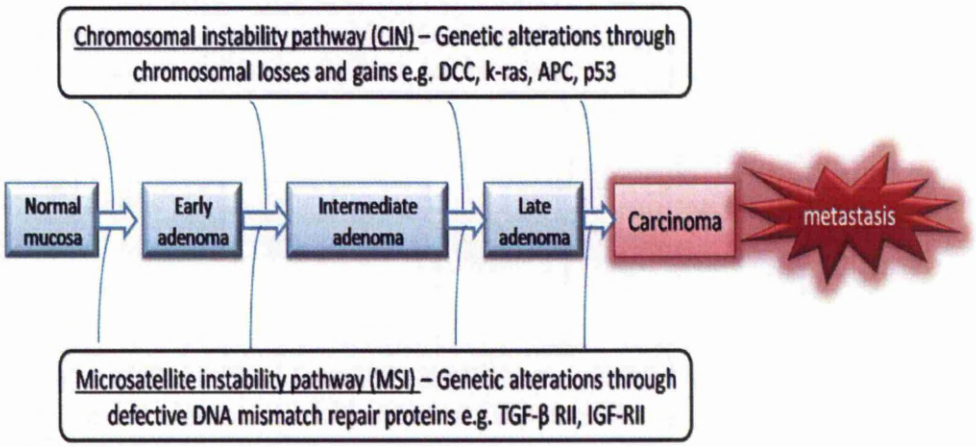


Figure 1: Genetic instability in colorectal cancer. APC, adenomatous polyposis coli; DCC, deleted in colorectal cancer; IGF-IIR, insulin-like growth factor II receptor; TGF-RII, transforming growth factor II receptor[11].

Genetic mutations result in histological transition from normal mucosa to adenomatous polyps, which are non-obligate precursors of malignant lesions. Adenomatous polyps account for half to two-thirds of colorectal polyps and are found in about a quarter of people by the age of 50 years[15]. The relationship between the characteristics of adenomas and the risk of cancer has been established by a British study of 1618 patients, which showed that individuals with tubulo-villous, villous or large (> 1 cm diameter) adenomas were more than three times more likely to develop colonic cancer than the general population. In the case of multiple recto-sigmoid polyps with advanced pathology, the risk of developing cancer increases to six times[16]. The concept of an adenomatous-carcinoma sequence in CRC has also been derived from indirect evidence as follows:

- Cancers and adenomatous polyps have a similar anatomic distribution in the large intestine[17].
- Large adenomas display greater cellular atypia and genetic mutations than small adenomas[18].
- Patients with one or more large polyps of more than 1 cm diameter have been found to be at increased risk of future CRC with cancers arising at the site of polyps left in place[19].
- Removing adenomas significantly reduces the incidence of CRC[20].
- Patients with FAP have hundreds to thousands of adenomatous polyps which are pre-malignant and share similar histological features to those of sporadic adenomas[21].

In addition to the conventional-type adenoma-carcinoma sequence following chromosomal instability pathway described above, the serrated pathway has been recognised as a separate entity in colorectal tumourigenesis. It is associated with somatic *BRAF* mutation and widespread hypermethylation of CpG islands in gene promoters[22]. Sessile serrated adenomas (SSAs) are the pre-malignant lesion of the serrated pathway. These lesions show distinct histological features and proximally located in the right colon[22].

FAP is an autosomal dominant disorder caused by a mutation in the tumour suppressor gene, APC, located on chromosome 5q21[23]. Patients with FAP have hundreds to thousands of adenomas in the colon and rectum, usually starting in adolescence. Almost all of these patients get CRC at around 40 years old, significantly younger than the sporadic CRC population. Less than 1 % of CRC only is caused by FAP.

Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is the most common form of hereditary CRC, accountable for 1-5 % of all CRC. It is inherited autosomal dominantly and carries an 80 % risk of colorectal cancer and a 50-60 % risk of endometrial cancer in women[24]. At least 5 genes have been identified implicated in HNPCC, with MSH2 on chromosome 2p and MLH1 on chromosome 3p responsible for the majority of HNPCC[23]. All of the HNPCC genes are named mismatch repair (MMR) genes, demonstrating microsatellite instability.

There are other rare inherited conditions associated with CRC, such as the Peuz-Jeghers Syndrome and Familial Juvenile Polyposis. The genes implicated in all of the above conditions are summarised in Table 1.

Hereditary condition	Genes implicated
Hereditary Non-Polyposis Colorectal Cancer (HNPCC)	MLH1, MSH2, MSH6, PMS1, PMS2
Familial Adenomatous Polyposis (FAP)	APC
Peuz-Jeghers Syndrome	STK11
Familial Juvenile Polyposis	PTEN, SMAD4, BMPR1A

Table 1: Inherited predisposition to CRC, summarised from Gatalica and colleagues[24].

1.1.2.2 Family history other than the hereditary conditions

Having a family history of CRC other than the hereditary conditions mentioned above, increases the risk to an individual developing the disease, possibly due to the interplay between environmental and genetic factors. Having a single

relative with CRC increases the risk of developing this disease 2-3 fold among the first degree relatives[25]. The finding of more numbers of closely related individuals increases the risk to their relatives further. If the single affected relative is young, for example, under 40 years old, the risk increases to 5 folds. In the majority of families with a history of CRC, lower penetrance genes may play a role, although the mechanism of cancer susceptibility in this group of patients remains poorly understood[25].

1.1.2.3 Inflammatory bowel disease (IBD)

Patients with IBD are at higher risk of CRC than the general population. Cancers that develop in IBD do not arise from adenomatous polyps but from areas of pre-cancerous dysplasia due to chronic inflammation. This causative factor is further described in chapter 1.2.

1.1.2.4 Metabolic syndrome

It has long been observed that CRC is associated with hypercholesterolaemia, obesity and diabetes mellitus, which fulfil the criteria for metabolic syndrome[26]. The National Cholesterol Education Program's Adult Treatment Panel III defined metabolic syndrome as having three or more of the criteria[27]: hypertension, central body adiposity, low high-density lipoprotein cholesterol, hypertriglyceridaemia and impaired glucose tolerance.

Several large cohort and cross-sectional studies have found association between CRC and metabolic syndrome, in which either the metabolic syndrome or its components increase the risk for CRC by approximately 50 %[28-31]. This risk seems to be higher in men than in women.

It is also interesting to note that several studies have reported the association between coronary artery diseases with advanced CRC. Chan and colleagues[32] reported that coronary artery disease remained associated with advanced CRC after adjusting for several confounding factors. Also, Limburg and colleagues[33] and Chung and colleagues[34] have found increased risk of CRC among diabetic patients. Furthermore, the risk of recurrent CRC increases in patients with insulin resistance disorder[35]. It is therefore apparent that the association of coronary artery disease with CRC described above shares several of the common criteria in defining metabolic syndrome. However, the strength of the association would need to be further evaluated following adjustment for many other confounding factors. More clinical and laboratory studies are needed to elicit the mechanisms of each of the independent factors in the metabolic syndrome leading to CRC. These undeniably important risk factors to CRC can then be incorporated into clinical practice in risk stratification for inviting patients for CRC screening programme.

1.1.2.5 Cigarette smoking

Numerous epidemiologic studies dated from the early twentieth century have investigated the association between cigarette smoking (CS) and CRC risk. In a review analysis by Giovannucci[36], all the studies conducted in 1950s and 1960s on men and women, who have not exceeded four decades of CS failed to show any significant association with CRC. However, studies conducted in US after 1970s, when most smokers have exceeded four decades of CS, showed that smoking significantly increases the risk of CRC, suggesting a long time lag of up to 35-40 years for CS to be accountable for increased risk for CRC.

Giovannucci concluded that 1 in 5 cases of CRC is attributable to tobacco use in the United States. A more recent meta-analysis consisting of 42 observational studies showed that CS seems to be associated with increased risk of colorectal adenoma (a precursor lesion for colorectal cancer development), including lesions with advanced clinico-pathological features[37]. To establish cause and effect between CS and CRC, two case-control studies have shown that CS was differentially associated with tumours that exhibit a high level of MSI, *BRAF*-mutated status, or the CpG island methylator phenotype[38, 39]. Further studies are needed to clarify the molecular pathways concerning tobacco-derived carcinogens and the development of CRC.

1.1.3 Clinical presentation and screening

The common clinical presentations of CRC patients include changes in bowel habits, iron deficiency anaemia, weight loss, abdominal pain, per-rectal bleeding, abdominal or rectal mass, or being acutely unwell with bowel obstruction or perforation. Generally, the symptoms are related to the site of CRC, e.g. iron deficiency anaemia and abdominal mass are more common in right-sided tumour, whereas fresh per-rectal bleeding is more common with recto-sigmoid cancer. However, all these are non-specific signs and symptoms of CRC as they are common in numerous other bowel pathologies.

Unfortunately, patients with CRC may have no clinical signs or symptoms until the late stage of the disease. Approximately 55 % of all patients diagnosed with CRC present with lymph node or distant metastases at the time of diagnosis with significantly lower survival rates[40]. Therefore, early detection before the presentation of clinical signs and symptoms is essential to reduce mortality.

CRC development is a process that spans many years, thereby providing an opportunity for early detection or prevention with a screening programme. In the UK, the National Health Service Bowel Cancer Screening Programme commenced in 2006 and invites men and women aged 60-69 (to 75 in 2010) to participate via submission of a faecal occult blood test (FOBT) every 2 years. Those with a positive FOBT are offered colonoscopy as a second line screening[41]. There are two types of FOBTs: the guaiac test which is based on the peroxidase-like activity of haem in haemoglobin and the immunochemical test which detects the globin moiety in haemoglobin. The guaiac-based FOBT has been adopted as the screening test because it has been shown to reduce both the incidence and mortality of CRC by ~20% and ~16% respectively[42]. The FOBT has several advantages: it is non-invasive, examines the entire colorectal tract, can be carried out in the privacy of one's own home, requires no patient preparation, and is simple and affordable. However, it suffers from the disadvantages of having low sensitivity and specificity for both adenoma and CRCs. In addition, the ingestion of certain foods (red meats, fruits and vegetables) and medicines (non-steroidal anti-inflammatory drugs) can yield false positive results[42].

1.1.4 Pathological staging, survival and other pathological prognostic factors

CRC staging system is an essential tool for diagnostic, predictive and prognostic purposes of the disease course. It is also paramount for tailoring appropriate patient management and meaningful clinical research. The late English physician and pathologist, Cuthbert Dukes (1890 – 1977) set a

standard in surgical pathology in 1932, when he established that the wall penetrance and nodal involvement were the most important factors in staging rectal cancer for determining clinical outcome[43]. The original Dukes' classification was divided into three simple stages, A to C (Table 2), and described the staging for rectal cancer only but it also applies to colonic cancer. Dukes' classification was then modified by Astler and Coller in 1954[44], followed by the introduction of the tumour, node, metastasis (TNM) staging by the American Joint Committee on Cancer (AJCC) in 1957.

Stage	Definition
A	Invasive carcinoma not breaching the muscularis propria.
B	Invasive carcinoma breaching the muscularis propria, but not involving regional lymph nodes.
C1	Invasive carcinoma involving the regional lymph nodes (apical node negative).
C2	Invasive carcinoma involving the regional lymph nodes (apical node positive).

Note: Dukes' stage D was added later to mean the presence of distant metastases.

Table 2: Dukes' staging definitions[45].

The TNM staging system (Table 3) of the AJCC and the International Union Against Cancer (UICC) is now the standard for colorectal cancer staging in the UK and United States[46]. It is internationally adopted due to its comprehensive set of definitions that ensure uniform use by multidisciplinary members, and is continuously improved upon through expert review of existing data[47]. However, Dukes' classification is still widely used owing to its simplicity and reproducibility. The staging systems illustrate that there are high percentages of

permanent cure for CRC in the early stages of the disease, with poor survival when the disease involves lymph nodes (Table 4).

Apart from the tumour (T), nodal (N) and metastatic (M) pathological features, there are other pathological prognostic factors that predict outcome in CRC. The microscopic grade, or degree of tumour cell differentiation, is an independent predictor of survival, with higher grades being associated with increasing wall penetration and nodal or distant metastasis and therefore worse prognosis[48]. Lymphatic vessel invasion[49], blood vessel invasion[50] and perineural invasion[51] have also been associated with poorer prognosis. On the other hand, peritumoural lymphocytic infiltration[52] and the observation of sinus histiocytosis and paracortical immunoblastic activity in the lymph nodes draining the tumour[53] have been reported to correlate with improved survival.

Molecular features of CRC also provide prognostic information. Patients with tumours possessing a high degree of MSI have a favourable prognosis than those patients who tumours are microsatellite stable[54]. In addition, loss of heterozygosity at chromosome 18q is associated with a worse prognosis, possibly due to the loss of the tumour suppressor gene – deleted in colon cancer (DCC)[55].

	Category	Definition
Primary tumour (T)	Tx	Primary tumour cannot be assessed.
	T0	No evidence of primary tumour.
	Tis	Carcinoma in situ (intraepithelial or intramucosal carcinoma).
	T1	Tumour invades the submucosa.
	T2	Tumour invades the muscularis propria.

	T3	Tumour invades through the muscularis propria into the subserosa or non-peritonealised pericolic or perirectal tissues.
	T4	Tumour directly invades other organs or structures (T4a) or perforates the visceral peritoneum (T4b).
Regional lymph nodes (N)	Nx	Regional lymph nodes cannot be assessed.
	N0	No regional lymph nodes metastasis.
	N1	Metastasis in one to three lymph nodes.
	N2	Metastasis in four or more lymph nodes.
Distant metastasis (M)	Mx	Presence of distant metastasis cannot be assessed.
	M0	No distant metastasis.
	M1	Distant metastasis.

Table 3: AJCC/UICC TNM definitions[46].

TNM				Modified Astler-Coller	Dukes	Frequency	5-year survival
Stage 0	Tis	N0	M0	N/A	N/A	N/A	N/A
Stage I	T1	N0	M0	Stage A	A	11%	80-85 %
	T2	N0	M0	Stage B1			
Stage IIA	T3	N0	M0	Stage B2	B	35%	72-75 %
Stage IIB	T4	N0	M0	Stage B3			65-66 %
Stage IIIA	T1, T2	N1	M0	Stage C1	C	26%	55-60 %
Stage IIIB	T3, T4	N1	M0	Stage C2, C3			35-42 %
Stage IIIC	Any T	N2	M0	Stage C1, C2, C3			25-27 %
Stage IV	Any T	Any N	M1	Stage D	D	29%	0-7 %

Table 4: CRC staging grouping of various classification systems with frequency and 5-year survivals[46, 56].

1.1.5 Biomarkers in CRC

Over the years, various research groups have performed genomic and proteomic analysis on CRC tissues, serum and faeces in order to identify biomarkers which could improve outcomes for CRC patients, by finding markers for early detection (diagnostic markers), prognosis (prognostic indicators), tumour responses (predictive markers), and disease recurrence (monitoring markers).

The Cancer Research UK (CR-UK) describes several stages in prognostic/predictive biomarker discovery (Figure 2)[57], which begins with identifying the rationale and clinical need of a biomarker in a cancer type, followed by assay development to assess the potential biomarker, and ultimately validate the correlation between the biomarker and clinical outcome in prospective randomised clinical trial before transferring the biomarker to routine clinical practice.

Kuramitsu and colleagues[58] in their comprehensive review on proteomic analysis on CRC tissues, have compiled a list of proteins that are up-regulated or down-regulated in CRC tissues. Despite extensive lists of proteins, the lack of sensitivity and specificity, or lack of thorough validation has precluded their use in clinical setting at present. Likewise, there is insufficient data to recommend the routine use of p53, thymidylate synthase, dihydropyrimidine dehydrogenase, thymidine phosphorylase, 18q loss of heterozygosity, or deleted in colon cancer (DCC) protein in the management of patients with CRC[59].

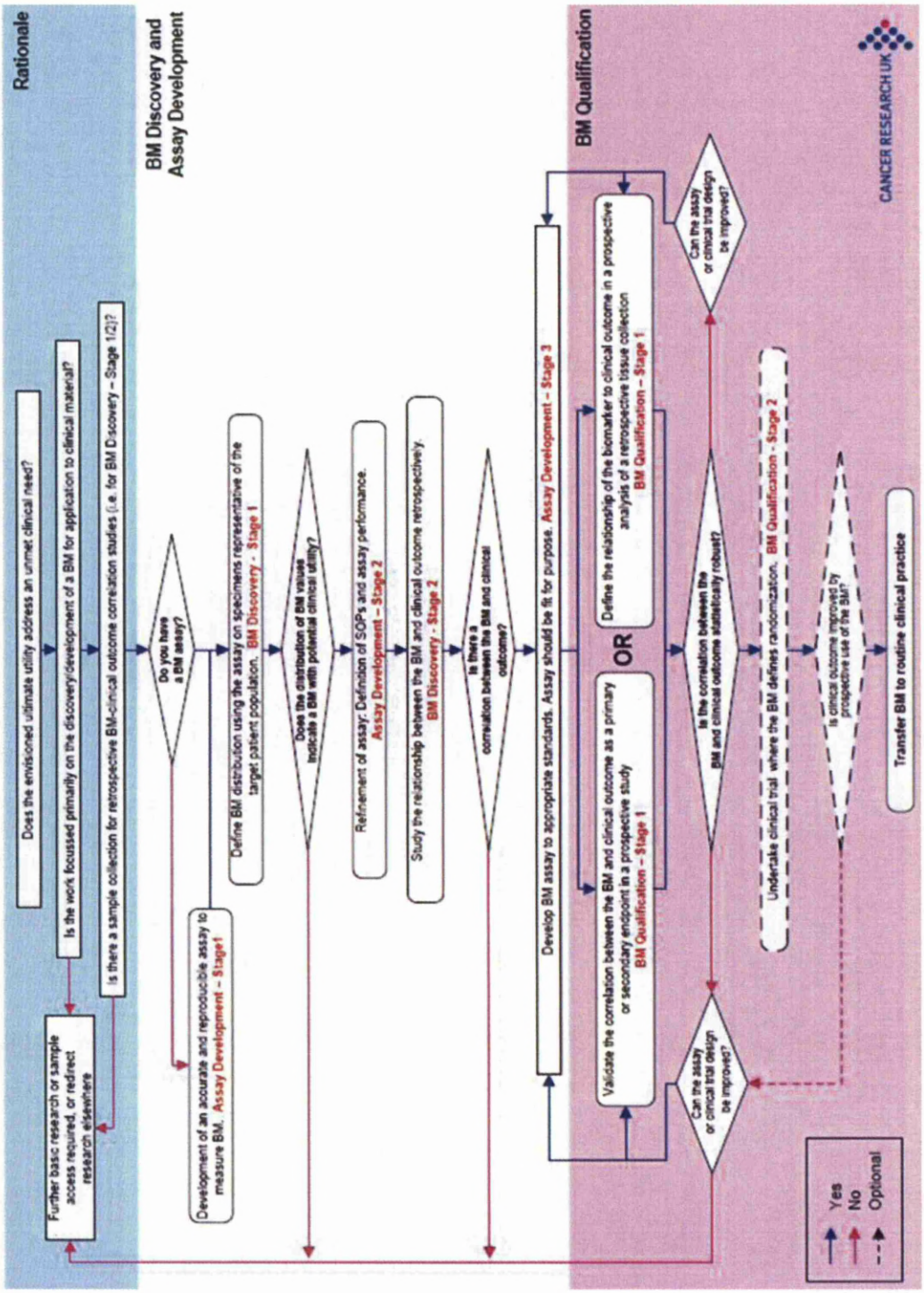


Figure 2: The Cancer Research UK (CRUK) prognostic/predictive biomarker roadmap[57].

However, several existing serum and tissue markers have been recommended by the European Group on Tumour Markers (EGTM)[60] and the American Society of Clinical Oncology (ASCO)[59] for routine clinical use: serum carcinoembryonic antigen (CEA), FOBT, and several genetic tests for genetic susceptibility to CRC such as APC for identifying subjects at high risk of developing FAP, and MSI/MLH1/MSH2/MSH6 for identifying subjects at high risk of developing HNPCC.

Serum CEA remains the oldest and the most widely used biomarker in patients with CRC. EGTM and ASCO recommend the use of serum CEA as a surveillance following curative resection or monitoring for the response to therapy in advanced disease. Several meta-analyses[61-64] have reported that the use of an intensive follow-up regimen resulted in a modest improvement in outcome compared to minimal follow-up regimen. Specifically, using serial CEA measurement had a significant impact on improved survival compared to not using CEA[61, 63]. Moreover, serum CEA measurement is inexpensive and causes minimal inconvenience for patients. However, it is not recommended for use as a screening test due to its poor sensitivity despite the specificity for CRC identifying occult CRC being high[65].

Perhaps, one of the most significant recent developments with respect to biomarkers in this field has been the observation that the presence of mutant *KRAS* in tumours is predictive of failure to derive treatment benefit from EGFR-targeted agents in patients with metastatic colorectal cancer[66, 67]. This leads to the use of EGFR-targeted agents, for instance, cetuximab as approved by the National Institute for Health and Clinical Excellence (NICE) for patients

with wild-type *KRAS*, non-resectable, liver-only metastatic colorectal cancer in the UK[68].

In addition, there are evidence that patients who have Stage II colorectal tumours with high-frequency MSI do not derive benefit from adjuvant 5-FU[69], and the current expert opinion is that prior testing of MSI status of these patients could be useful to determine which of these patients should be treated with adjuvant 5-FU[70].

1.1.6 Management of CRC

1.1.6.1 Surgery

Surgery remains the definitive treatment for localised CRC, offering the only chance of cure. This involves complete resection of the primary tumour with adequate margins (distal margins of at least 5 cm), including the artery supply, venous drainage and lymph node groups that drain the diseased segment. At least 12 lymph nodes should normally be harvested and examined to allow accurate staging of the tumour[2]. In rectal cancer surgery, total mesorectal excision, which involves the removal of the tumour as well as the fatty tissue around the rectum, has become the standard procedure for mid- and low rectal tumours. It results in higher local control and increased disease-free survival[71].

The liver is a major site of distant metastasis in colorectal cancer. It has been reported that as many as 25 % of CRC patients have liver metastasis at initial presentation, and a further 20-30 % of patients develop metachronous disease following colorectal cancer surgery[72]. Of these patients, only about 20 % would be amenable to curative resection of liver metastasis which substantially

improves 5-year survival rates of up to 37-58 %[72]. A variety of therapeutic approaches have been employed to improve liver surgery and increase number of patients undergoing treatment of liver metastasis, which include pre-operative embolisation or chemotherapy, cryotherapy, radiofrequency ablation of small lesions and two-stage liver resection[70].

1.1.6.2 Neo-adjuvant and adjuvant therapy

For the last two decades, 5-fluorouracil (5-FU) has formed the basis and the mainstay of therapy for CRC, with the developments revolving around 5-FU in search of optimum combination regimens. Recently, the arrival of two new drugs, oxaliplatin and irinotecan, has provided promising outcomes in treating CRC either as an independent agents or in combination regimens. Specifically to rectal cancer, radiotherapy has been the gold standard treatment. Chemotherapy and radiotherapy can be given either pre-operatively (neo-adjuvant) or post-operatively (adjuvant).

1.1.6.3 Fluorouracil-based therapy

Fluorouracil is a fluorinated pyrimidine that acts through inhibition of thymidylate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis[73]. It induces cell cycle arrest and leads to apoptosis by inhibiting the ability of cells to synthesise DNA.

In the early 1990s, a large clinical trial conducted by the Eastern Cooperative Oncology Group which involved 1296 patients showed that adjuvant treatment containing 5-FU significantly reduced the risk of disease recurrence by 41 % and the risk of death by 33 % compared to surgery alone in stage III disease but

not in stage II disease[74, 75]. Therefore, the National Cancer Institute Consensus Conference recommended the use of fluorouracil-based adjuvant therapy as standard of care in patients with resected stage III colon cancer[76]. Later, leucovorin (LV, also called folinic acid), a reduced folate has been shown to enhance the anti-tumour effect of 5-FU by stabilising the interaction of fluorouracil with thymidylate synthetase[77]. The 5-FU/LV combination produced an increase in 5-year disease-free survival from 42 % to 58 % and 5-year overall survival from 51 % to 61 % in stage III disease[78], which is now the routine treatment regimen.

Various administration schedules in terms of lengths of treatment, dosages, routes of administration of 5-FU/LV have also been investigated (Table 5)[79]. The use of oral 5-FU, capecitabine, has been shown to confer similar efficacy to infusional 5-FU[80]. de Gramont regimen is one of the most widely used regimens in Europe. The Medical Research Council (MRC) CR06 trial showed that there was no significant difference in efficacy between de Gramont, Lokich or raltitrexed regimen, but raltitrexed regimen resulted in more serious adverse events[81]. Therefore, the infusional 5-FU/LV for 6 months remains the regimen of choice in the UK. However, the choice of regimen ultimately depends on variable side effect profiles between individual patients. For example, the Mayo Clinic regimen frequently causes neutropenia and stomatitis, and the Roswell Park regimen can result in debilitating diarrhoea. The continuous infusion regimen (e.g. Lokich) can be associated with less haematological and gastrointestinal toxicity, but more commonly related to hand-foot syndrome[79].

Although fluorouracil-based adjuvant therapy produces great survival benefits to patients with stage III disease, many trials failed to demonstrate such benefit in patients with stage II colon cancer. A systemic review which included 37 trials and 11 meta-analyses concluded that adjuvant therapy is associated with only a small disease-free survival but no overall survival benefits for patients with stage II colon cancer[82]. This is consistent with the findings from a recent meta-analysis of The Cochrane Collaboration[83]. However, a large trial conducted by a Birmingham (UK) group (Quick and Simple and Reliable study – QUARSAR) found that in patients with stage II CRC, adjuvant 5-FU/LV incurs small absolute survival improvements, by assuming that the 5-year mortality without chemotherapy is 20%[84]. Therefore, it seems essential to discuss the benefits of adjuvant systemic chemotherapy with stage II cancer patients, taking into considerations the co-morbidities and likelihood of side effects to allow informed decision to be made.

Name	Country of research group	Regimen
Mayo	USA	Bolus low-dose LV 20 mg/m^2/day followed by 5FU 425 mg/m^2/day given daily days 1-5, every 28 days
De Gramont	France	LV 200 mg/m^2 2-hour infusion, 5-FU bolus 400 mg/m^2 and 5-FU 600 mg/m^2 22-hour infusion, days 1 and 2, 14 days
Modified De Gramont	UK	LV 175 mg (flat dose) 2-hour infusion, 5-FU bolus 400 mg/m^2 and 5-FU 2,800 mg/m^2 46-hour infusion, every 14 days
Lokich	UK	Continuous infusion 5-FU 300 mg/m^2/day (10

		weeks or more)
AIO	Germany	LV 500 mg/m^2 + 5-FU 2,600 mg/m^2/24-hour infusion, weekly x 6, every 8 weeks
Roswell Park	United States	LV 500 mg/m^2 2-hour infusion followed by 5-FU 500 mg/m^2 bolus 1 hour after the start of LV infusion, weekly x 6, every 8 weeks

Table 5: The commonly used 5-FU regimen[79].

1.1.6.4 Irinotecan

Irinotecan is a semi-synthetic derivative of the natural alkaloid camptothecin. Its active metabolite SN38 causes cytotoxicity by inhibiting topoisomerase I, an enzyme that catalyses breakage and rejoining of DNA strands during DNA replication[85]. There is good clinical evidence that irinotecan as a single agent[86], or used in combination with 5-FU[87, 88], is an effective second-line treatment of patients with 5-FU resistant disease or as first line in metastatic disease. However, the addition of irinotecan to 5-FU/LV as first line adjuvant treatment (IFL regimen) following curative resection of stage III disease has been shown to incur increased toxicity and confer no improvement in outcome[89, 90]. Therefore, the use of irinotecan is currently limited to patients with advanced disease.

1.1.6.5 Oxaliplatin

Oxaliplatin is a diaminocyclohexane platinum compound that forms DNA adducts in a similar manner to cisplatin, leading to impaired DNA replication and cellular apoptosis[91]. Oxaliplatin treatment benefits patients with metastatic CRC when it is administered with 5-FU/LV but not as single agent[92, 93]. The FOLFOX regimen which involved infusional 5-FU, LV and oxaliplatin

improved overall survival significantly in metastatic disease when compared with IFL regimen or a combination of irinotecan and oxaliplatin[93]. This finding has led to the investigation of FOLFOX regimen in patients with stage II and stage III diseases. The Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer study[94] and the National Surgical Adjuvant Breast and Bowel Project-07 trial[95] found that adding oxaliplatin to 5FU/LV significantly improved disease-free survival and overall survival in the adjuvant treatment of stage III colon cancer. Therefore, FOLFOX regimen is now considered the standard of care for completely resected colon cancer in fit and young patients.

1.1.6.6 Chemo-radiation in rectal cancer

In patients with rectal cancer, distant metastasis is the most common cause of death. Patients with loco-regional and pelvic recurrences suffer from severe sacral or deep pelvic pain which is associated with significant morbidity and poor quality of life. Therefore, unlike colon cancer, loco-regional recurrence is also a paramount end point in the treatment of rectal cancer.

The benefits of radiotherapy in rectal cancer were established in the late 1980s. Studies have shown that combined adjuvant radiotherapy and 5-FU therapy resulted in a significant reduction in the rate of local recurrence and an increase in the rate of overall survival when compared with surgery alone[96, 97].

Later, pre-operative chemoradiotherapy for rectal cancer has also been extensively investigated because of certain advantages over post-operative therapy as below:

- Capable of down-staging the tumour, improving surgical resectability, and ultimately increasing curative surgical resection with adequate margins[98].
- Allows tumours to be resected with limited longitudinal surgical margins, hence increasing the level to which sphincter-sparing procedures can be performed in the distal rectum instead of abdomino-perineal resection that leads to permanent colostomy[99].
- Associated with reduction in tumour spillage and the potential for dissemination of tumour cells during surgery, thus leading to lower risk of recurrence[100].
- Before surgery, the tumour bed blood supply is well preserved. This enhances oxygenation, thereby improving irradiation efficacy[101].
- Reduced risk of chemoradiation-induced morbidity, for instance radiation enteritis, because small bowel is less likely to be adherent in the treatment field prior to surgery[98].

The German Rectal Cancer Study Group conducted a large clinical trial, in which 823 patients with clinical stage T3 or T4 or node-positive rectal cancer were randomised to receive either the pre- or post-operative chemoradiotherapy[102]. This study demonstrated that pre-operative treatment had doubled the rate of sphincter-sparing operations and reduced significantly the rates of local recurrence, acute toxicity, and long-term toxicity compared to post-operative treatment. However, there was no difference in disease-free or overall survival between the two treatment arms. Furthermore, the Swedish Rectal Cancer Trial[103] and the MRC CR07/NCIC-CTG C016[104] have shown that pre-operative radiotherapy in resectable rectal cancer significantly reduced

the rates of local recurrence compared with post-operative chemoradiotherapy. Given these compelling results, following pre-operative MRI assessment of the circumferential resection margin, patients undergo surgery alone or treated with neo-adjuvant short-course radiotherapy (25 Gy in 5 fractions) if this margin is likely to be negative at the time of surgery. If this margin is likely to be positive or in node-positive disease, then patients are managed with long-course neo-adjuvant chemoradiation (50.4 Gy in 28 fractions)[70].

1.1.6.7 New therapeutic agents for CRC

1.1.6.7.1 Epidermal growth factor receptor (EGFR) inhibitors

The Human EGFR (erbB, or HER) family plays an important role in cellular growth, proliferation and apoptosis. In CRC, EGFR expression has been demonstrated in up to 70 % of tumours[105] and these tumours carry a poorer prognosis[106]. Cetuximab and panitumumab are monoclonal antibodies targeting EGFR specifically, both licensed for use in patients with advanced, wild-type *KRAS* colorectal cancer after failure of 5-FU, oxaliplatin and irinotecan-containing regimens. Pre-clinical models have shown an additive effect of cetuximab and irinotecan in tumour cells sensitive to and resistant to irinotecan[107]. A phase II study carried out in patients with metastatic disease, refractory to irinotecan showed that the combined treatment of cetuximab and irinotecan resulted in an increased response rate[108]. A further trial has confirmed the effective, synergistic activity of cetuximab and irinotecan, as cetuximab itself is markedly ineffective[109]. The use of anti-EGFR in adjuvant setting, and in combination with 5FU/leucovorin/oxaliplatin in pre-operative

setting among patients with curative, resectable colon cancer (known as the FOxTROT trial) is currently under examination.

1.1.6.7.2 Angiogenesis inhibitors

Vascular endothelial growth factor has been identified to be the predominant angiogenic factor in human CRC and its high expression is associated with metastases and poor prognosis[110]. Bevacizumab is a humanised monoclonal antibody directed against vascular endothelial growth factor. Its anti-tumour effect has been investigated in patients with metastatic disease, in combination with 5-FU/LV[111], irinotecan/5-FU/LV[112], or FOLFOX regimen[113]. These trials demonstrated that bevacizumab, when used in combination with other chemotherapy improves survival in patients with metastatic CRC. Like cetuximab, the role of bevacizumab in adjuvant setting of curative resected CRC is under examination.

1.2 The link between inflammation and cancer

The link between inflammation and cancer has been explored. In fact, this association was first hypothesised in 1863 by the late German physician and pathologist, Rudolph Ludwig Carl Virchow (1821-1902), who observed the presence of inflammatory cells in biopsied samples from tumours, and cancers frequently took place at sites of chronic inflammation[114]. However, his observation was not taken in seriously until the report by Harold Dvorak in 1986, which noted that the tumour stroma and inflammatory structure in wound healing shared similarities. This prompted him to refer to tumours as 'wounds that do not heal'[115]. Recent years of cancer research have seen the renaissance of

Virchow's hypothesis with mounting evidence which contributes to the understanding of the cellular and molecular mechanisms in cancer-related inflammation (CRI). While the renowned six hallmarks of cancers (self-sufficiency of growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and the ability to invade tissues and metastasise) were described by Hanahan and Weinberg[116] approximately a decade ago, the bearing of CRI as the seventh hallmark of cancer has only been acknowledged and generated great interest recently[117].

Balkwill and Mantovani have summarised the evidence from epidemiological and laboratory studies that links several types of cancer and inflammation (see review[118]). In colorectal, this association was apparent among patients with inflammatory bowel disease i.e. ulcerative colitis (UC) and Crohn's disease, that they inherit approximately 2-3 folds risk than the general population of developing CRC[119]. A meta-analysis study including 116 studies by Eaden and colleagues[120] showed that the estimated risk of CRC in patients with UC to be 3.7% at 10 years. However, more recent studies reported the risk of CRC in patients with inflammatory bowel disease to be much lower than that reported by Eaden et al's meta-analysis. For instance, Rutter and colleagues[121] from St. Mark's Hospital in the United Kingdom reported that the cumulative incidence of CRC in UC patients was 2.5% at 20 years, 7.6% at 30 years and 10.8% after 40 years of disease. In addition, a population based study in Hungary showed the cumulative incidence of CRC was 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years of chronic UC[122]. Furthermore, studies from Denmark and the Mayo clinic found no increased risk between UC and CRC when compared to the general population[123, 124]. The observed change in incidence could be due

to the result of increased colonoscopic surveillance and improved medical and surgical treatments among patients with inflammatory bowel disease. Despite this, the link between CRC and chronic inflammation in inflammatory bowel disease is supported by the evidence that the risk of CRC increases with higher degree of inflammation: early age at the diagnosis of inflammatory bowel disease, long duration of symptoms, and the full extent of the disease anatomically such as pancolitis which features severe inflammation in the whole of colon with high prevalent of dysplasia-carcinoma cascade[125].

Perhaps, the most direct evidence linking the significance of inflammation and colorectal cancer comes from studies investigating cancer risk in relation to the long term use of non-steroidal anti-inflammatory drugs (NSAIDs). Several epidemiological studies have reported an inverse relationship between colorectal cancer risk and aspirin use[126-129]. Moreover, randomised clinical trials have shown that NSAIDs are effective in the primary prevention of CRC[130, 131] and regular use after the diagnosis of CRC is associated with lower risk of mortality[132]. This is further supported by experimental *in vivo* studies in a murine model of familial adenomatous polyposis, NSAIDs had been shown to inhibit tumour formation and caused regression of existing tumours by inhibiting cyclo-oxygenases (COX-1 and -2) which are essential factors in the inflammatory cascade[133, 134].

However, the above clinical studies did not investigate the potential hazards of long term aspirin in comparison with its benefits in chemo-prevention of colorectal cancer. Also, in the presence of alternative, effective prevention strategy (The UK National Bowel Cancer Screening Programme) for colorectal

cancer, the necessity and benefits of long term use of aspirin is unknown. With these uncertainties, aspirin is currently not recommended for the general population for colorectal cancer prevention.

There are two pathways which depict CRI[118, 135]: the extrinsic pathway, also known as inflammation-induced cancer, driven by chronic inflammatory process such as that in inflammatory bowel disease which promotes carcinogenesis; and the intrinsic pathway, or cancer-induced inflammation, arises from genetic alterations such as oncogenes leading to tumour-mediated host inflammatory response in the tumour microenvironment. Either way, numerous inflammatory mediators which are normally expressed or released in response to cellular stress or damage/necrosis, known as the endogenous damage-associated molecular pattern molecules (DAMPs) or alarmins, have been implicated in tumour development and progression[135].

In our Research Unit, at the Division of Surgery and Oncology, we described high level expression of stromal S100A8/A9 proteins and their association with tumoural levels of the tumour suppressor protein Smad4[136, 137]. In a separate study, the prognostic value of epithelial HSP27 protein expression in rectal tumours was elucidated[138]. Both S100A8/A9 and HSP27 proteins are endogenous DAMP molecules and elaborating their functions in colorectal cancer formed the basis of the research undertaken for my project.

1.2.1 The endogenous damage-associated molecular pattern (DAMP) molecules

The noted immuno-biologist at Yale University Medical School, the late Charles Alderson Janeway, Jr (1943-2003) proposed that the immune system of multi-cellular organisms protects the host from foreign infectious pathogens via evolved receptors of the antigen-presenting cells which recognise conserved products of microbial origins, also known as the pathogen-associated molecular patterns (PAMP) molecules and thus allow the immune system to distinguish infectious non-self from non-infectious self[139]. In the absence of the exogenous PAMP molecules, robust immune responses are also elicited against several conditions such as tissue transplants, tumours, autoimmune diseases etc on the basis of detecting and protecting the host against endogenous danger, corresponding to the 'danger model' proposed by a scientist called Polly Matzinger in 1994[140]. Through pattern recognition as seen on the response to exogenous microbial molecules as well as a tissue-driven immune system in the absence of external factors, Matzinger then described the concept that antigen-presenting cells also respond to endogenous molecules released from cell stress, injury or necrosis which promote immune responses and inflammation[140]. Together, these endogenous molecules, also called alarmins, and PAMPs constitute the DAMPs[135].

The endogenous DAMPs are intracellular proteins, DNAs, RNAs or nucleotides which have been classified into various categories and described in detail by Rock and Kono[141]. DAMPs include High Mobility Group Box 1, S100 proteins, heat shock proteins (HSPs), uric acids and interleukins. Due to their particular

relevance to the research described in this thesis, the tumourigenic role of extracellular S100A8 and S100A9 and their part in the crosstalk between tumour and its stromal cells is described in section 1.22 of this chapter, whilst the role of intracellular HSP27 in cancer is described in section 1.23 of this chapter.

1.2.2 The crosstalk between tumour and its stroma

1.2.2.1 Tumour stroma/microenvironment

More than a hundred years ago, the renowned English surgeon Stephen Paget proposed the 'seed and soil' hypothesis to explain the non-random dissemination patterns of certain human malignancies, and that while many researchers have been studying 'the seeds', 'the soils' may reveal valuable insights into the 'metastatic peculiarities' of cancers[142]. Recent years have seen the hypothesis revisited following the recognition that the microenvironment of a developing tumour is a crucial regulator of carcinogenesis.

The tumour microenvironment consists of three main components[143]: insoluble extracellular matrix; stroma consisting of fibroblast, adipose, vasculature and resident immune cells; and the conventional milieu of cytokines and growth factors.

In normal circumstances, the complex interplay, known as the 'crosstalk' between parenchymal cells and their microenvironments is aimed at maintaining balanced homeostasis in tissue function. The disruption of the interplay between parenchyma and their microenvironment can induce aberrant cell proliferation, adhesion, function and migration that might promote formation of malignancy[144]. Studies have shown that changes in stromal

behaviour can promote epithelial transformation[145, 146]. Upon abnormal transformation, these cells may recruit and activate the vasculature and stroma through production and secretion of stimulatory growth factors and cytokines. In turn, the locally activated cellular and extracellular elements of the host microenvironment secrete molecules that can influence the malignant phenotype and modify the proliferative and invasive behaviour of the tumour cells through three mechanisms: increasing the genetic instability of tumour cells[147]; inducing signalling cascades in tumour cells via tumour-associated receptors thereby controlling gene expression in these cells; and by exerting selective pressures on the cells[148].

The crosstalk between tumour and its microenvironment is a bi-directional process and involves an extremely complex cohort of molecules. The understanding of such complex molecular crosstalk between tumour and its microenvironment may lead to the development of novel therapeutic agents in cancer management. Some of these well-characterised processes and molecules are summarised below:

a) Fibroblasts produce chemoattractants such as scatter factor/hepatocyte growth factor, which increases tumour cell motility by binding to the Met receptor[149].
b) Tumour cells secrete angiogenesis factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, which bind to receptors on stromal vascular cells, resulting in increased vascular permeability, endothelial proliferation, migration and invasion[150].

c) Fibroblasts and endothelial stromal cells produce enzymes including matrix metalloproteinases and urokinase plasminogen activator which bind on the surface of the carcinoma invadopodia and become activated, thereby degrading the extracellular matrix and clearing a pathway for tumour cell invasion[151].

d) Extracellular matrix degradation releases growth factors such as TGF-β and EGF, which then bind to receptors (TGF-βR and urokinase receptor) on the carcinoma cells[152].

1.2.2.2 The S100 proteins

S100 proteins are a family of highly acidic, low molecular weight proteins that were originally found in large concentration in the brain[153]. They constitute the largest group of calcium binding proteins. The name 'S100' derives from the property of these proteins being soluble in a 100 % saturated ammonium sulphate solution[153]. More than twenty S100 proteins have been identified to date. They not only display cell tissue and cell specific expression patterns, they are also present in extracellular space when they are thought to function like cytokines in regulating protein phosphorylation, calcium homeostasis, cytoskeletal dynamics, cell growth and differentiation, and the inflammatory response[154].

1.2.2.2.1 The structure and expression of S100A8 and S100A9 proteins

S100A8 (Myeloid-related Protein-8, calgranulin A) and S100A9 (Myeloid-related Protein-14, calgranulin B) are cytosolic molecules characterised by two EF hands. When illustrated diagrammatically, the EF hand motifs contains the helix-loop-helix conformation, where two alpha helices are positioned nearly

perpendicular to each other and linked by a short loop regions that binds calcium ions[155]. The S100A8 and S100A9 proteins can exist as homodimer, or form heterodimers, tetramers or oligomers depending on the presence of calcium[156, 157].

S100A8/A9 are inflammatory proteins constitutively expressed in myeloid cells, including monocytes and neutrophils and secreted at sites of inflammation[158]. It has been recognized that monocytes can be recruited from the blood circulation to the areas of inflammation or tumour microenvironment where they differentiate into macrophages[159]. While maturing and differentiating from monocytes into mature macrophages, their expression of S100A8 and S100A9 also change. Initially both of these proteins are expressed in the monocytes. As they mature, S100A8 expression diminishes leaving only S100A9, which is also subsequently lost as the cell matures further[160]. The differential pattern of S100A8 and S100A9 expression in macrophages has been shown to correlate with the severity of inflammatory process[161].

1.2.2.2.2 The functions of S100A8/A9

The importance of the expression of S100A8 and S100A9 in myelo-monocytic cell differentiation is unknown but generally it has been proposed that S100 proteins play a specific role in cell-cycle progression, and involved in cell maturation, differentiation and activation[162]. However, a functional role of intracellular S100A8 and S100A9 in phagocytes was established for the interaction with microtubules, although binding with other cytoskeletal intermediate filaments has also been described[163-165]. S100A8/A9 binds to tubulin in the presence of calcium and promotes tubulin polymerisation and

therefore stabilisation of tubulin filaments which is crucial for phagocyte migration[163]. Indeed, Vogl and colleagues[163] showed that the migration of phagocytes and wound healing ability were diminished in S100A9-/- mice that lack functional S100A8/A9 complex which resulted in significantly lower amount of polymerised and cytosolic tubulin.

Much of the functions of S100A8/A9 have been described as extracellular proteins. Rammes and colleagues[166] provided evidence that upon stimulation by inflammatory cytokines, S100A8/A9 were secreted actively by monocytes via an energy-dependent novel pathway following activation of protein kinase C. This specific process requires an intact microtubule network and is independent of the classical endoplasmic reticulum/Golgi route.

Upon secretion, S100A8/A9 binds with high affinity specifically to heparan sulphate proteoglycan[167] and carboxylated N-glycans[168] on enthothelial cells, or the receptor for advanced glycation end products (RAGE) which is expressed in many cell types including smooth muscle cells, monocytes, lymphocytes and endothelial cells. These bindings provoke the release of various inflammatory cytokines and adhesion molecules in orchestrating a pro-inflammatory and thrombogenic response. This was shown by Viemann and colleagues[169] in experiments using human microvascular endothelial cell lines and oligonucleotide microarray technology. They have also shown in *in vitro* experiments that the binding of S100A8/A9 to endothelial cells resulted in a loss of cell-cell contacts and an increased permeability of endothelial monolayer, which is a pre-requisite for leukocyte extravasation and recruitment of inflammatory cells from intra-vascular to extra-vascular space in

inflammation. In addition, S100A8 and S100A9 are potent chemokines for neutrophils[170], and they induce neutrophil adhesion to fibrinogen by activation of the β_2 integrin Mac-1[171]. These highlight the role of extracellular S100A8/A9 as an endogenous danger signal, which mimics endogenous toll-like receptor ligand involved in the inflammatory activation of phagocytes in response to the microbial lipopolysaccharides. Beside its pro-inflammatory property in combating infection, S100A8/A9 has also been shown to possess direct anti-microbial ability in inhibiting bacterial and fungal growth *in vitro* through zinc chelation[172-174].

With their roles in inflammatory process uncovered, S100A8/A9 has not only been perceived as a valuable biomarker of inflammatory processes, but also implicated in the pathogenesis of several chronic pathological inflammatory conditions such as rheumatoid arthritis, transplant rejection, systemic lupus erythematous, vasculitis, psoriasis etc[153, 175].

1.2.2.2.3 S100A8/A9 and cancer

The cluster of S100 genes is located at the chromosomal region of 1q21-q23, which is also been shown to be the common malignancy-associated regions of transcriptional activation frequently observed in human epithelial tumours[176]. Earlier proteomic studies using 2D-gel analysis and mass spectrometry revealed up-regulation of S100A8 in the pancreatic[177] and S100A8 and S100A9 in colorectal tumours[178]. S100A8 and S100A9 were also found to be present in cystic fluid from ovarian carcinomas and serum of the corresponding patients, but absent in fluid or serum from patients with benign ovarian cysts[179]. Using tissue microarrays and immunohistochemistry, Cross and

colleagues[180] reported the expression of S100A8 and S100A9 in various cancer types, on a very small number scales (n = 1-10 for each cancer types). Recently at the Division of Surgery and Oncology, University of Liverpool, in a more detailed analysis of protein expression profile of pancreatic tumours, Sheikh and colleagues[137] undertook 2-DE analysis of laser capture microdissected malignant and stromal components of tumours and benign ductal elements, and identified high levels of S100A8 and S100A9 proteins in tumour-associated stroma but not in benign or malignant epithelia. Furthermore, using IHC, S100A8 and S100A9 expression was observed in the stroma of myeloid cells of pancreatic tumours. Indeed, Stulik and colleagues[178] also reported that S100A8- and S100A9-positive cells were present in the stroma and invasive margin of colorectal carcinoma.

Associations between the expression of S100A8 and S100A9 and clinico-pathological parameters have also been reported in several cancer types (Table 6). In particular, over-expression of S100A9 in cancer cells has been associated with poor tumour differentiation in lung[181], breast[182], liver[183] and thyroid[184] adenocarcinomas. In prostate cancer, significantly higher levels of S100A9 have been reported compared to healthy controls or patients with benign prostate hyperplasia[185].

Cancer types	Authors	Findings
Pulmonary adenocarcinoma	Arai *et al.*, 2001[181]	S100A9 immunopositivity in pulmonary cancer cells correlated to poor tumour differentiation (n = 70). S100A8 expression was not examined.
Breast (invasive ductal)	Arai *et al.*, 2008[186]	S100A8 and S100A9 immunopositivity in ductal adenocarcinoma cells showed significantly: a) A positive correlation of immunoreactivity

		between S100A8 and S100A9. b) The percentage of S100A9-positive tumour cells was higher than that of S100A8-positive tumour cells c) Immunofluorescence showed S100A8 and S100A9 co-expressed, but S100A9 also observed in S100A8-negative cells. d) Each S100A8 and S100A9 correlated with the mitotic activity, HER2 overexpression, node metastasis, poor pT stage and overall pStage. e) Co-expression of both proteins was associated with tumour differentiation, vessel invasion, node metastasis, poor overall pStage (n = 101).
Breast (invasive ductal)	Arai *et al.*, 2004[182]	S100A9 immunopositivity correlated significantly with poor tumour differentiation, especially in nuclear pleomorphism and mitotic activity. No significant differences in the number of myelomonocytic cells expressing S100A9 (n = 70). S100A8 expression was not examined.
Hepatocellular adenocarcinoma	Arai *et al.*, 2000[183]	S100A9 immunopositivity correlated significantly with poor tumour differentiation grade (n = 70). No immunopositivity was observed in the normal hepatocytes and bile duct epithelia. S100A8 expression was not examined.
Prostate	Hermani *et al.*, 2005[185]	S100A8, S100A9 and RAGE were over-expressed in prostatic intra-epithelial neoplasia especially in high-grade adenocarcinomas. Benign tissue showed no or weak expression of the proteins. There was a high degree of overlap of the expression of these proteins. S100A9 serum levels were significantly elevated in cancer patients compared with benign patients or healthy individuals (n = 75)

Thyroid	Ito *et al.*, 2005[184]	S100A9 immunoreactivity correlated highly with undifferentiated carcinomas (n = 19). S100A8 expression was not examined.
Pancreas	Sheikh *et al.*, 2007[137]	Strong expression of S100A8 and S100A9 in stromal myeloid cells ($CD14^+/CD68^-$ monocytes) but not cancer cells. S100A8 co-expressed with S100A9. The presence of S100A8- and S100A9-positive cells did not correlate significantly with clinico-pathological parameters. The microenvironments of tumours which lacked Smad4 expression had significantly reduced numbers of S100A8-immunopositive but not S100A9-immunopositive cells.

Table 6: The expression of S100A8 and S100A9 in cancers and its clinical significance.

Using a proteomic-based approach, Mr Ilyas Khattak (an MD student) and Dr Taoufik Nedjadi (a Post-doctoral Research Associate) at the Division of Surgery and Oncology, University of Liverpool, investigated variation in the protein expression in primary CRC specimens of stage-matched patients. Proteins isolated from the stroma of twelve frozen colorectal cancer specimens were subjected to two-dimensional gel analysis followed by liquid-chromatography tandem mass spectrometry (LC-MS/MS), which resulted in the identification of differential expression of S100A8 protein in colorectal tumours (see Figure 1 of the published paper by Ang and colleagues[136] appended to this thesis). Immunohistochemistry (IHC) undertaken by Ms Elizabeth Tweedle (an MD student, Division of Surgery and Oncology, University of Liverpool) for the detection of S100A8 and its dimerisation partner, S100A9, revealed the expression of both S100A8 and S100A9 proteins

in cells scattered throughout the tumour stroma (see Figure 2A of Ang and colleagues[136]). Colorectal tumours generally expressed fewer S100A8-positive than S100A9-positive cells, and loss of Smad4 expression in primary colorectal tumour cells was associated with a significantly lower median count of S100A8-positive but not S100A9-positive stromal cells (see Figure 2C of Ang and colleagues[136]), as observed previously by Sheikh and colleagues[137] in pancreatic cancer. Furthermore, co-localisation of S100A8 or S100A9 was observed in some cells with the monocyte marker CD14 but not the macrophage marker CD68 (see Figure 2B of Ang and colleagues[136]), indicating that the S100A8/A9 population may be at an early stage of monocyte/macrophage maturation.

The degree of stromal S100A8/A9-positive infiltration showed no correlation with the parameters of age at surgery, gender, site of tumour, depth of tumour invasion or nodal metastases. However, high S100A9 cell counts were associated with poor differentiation grade ($P = 0.036$), and independently associated with large tumour sizes ($P = 0.02$) (see Supplementary Table 1 of Ang and colleagues[136]).

Neither S100A8-positive nor S100A9-positive stromal cell counts were associated with patient survival. Nevertheless, for the smaller cohort of patients exhibiting loss of Smad4 expression, high S100A8/A9 cell counts predicted poor 3-year survival, while no survival difference was observed for either high S100A8 or S100A9 counts at 60 months in this Smad4-negative cohort (see Figure 3 of Ang and colleagues[136]). This suggests that Smad4-negative tumours

have a shorter time to recurrence or metastases in the presence of high stromal S100A8- or S100A9-expressing monocytes.

The roles of S100A8 and S100A9 in cancer have also been examined in *in vitro* and *in vivo* experiments. Yui and colleagues[187, 188] reported the apoptotic activity of S100A8/A9 on tumour cells (mouse lymphoma and human leukaemia cells) and fibroblasts via a zinc-dependent mechanism. Further study by the group showed that S100A8/A9 bind directly to mammary carcinoma cells which resulted in the induction of apoptosis via a specific receptor-dependent mechanism[189]. Indeed, these findings have been supported by Ghavami and colleagues[190], who reported the apoptotic effect of S100A8/A9 on colonic carcinoma cell lines via zinc exclusion from the target cells and an undefined mechanism involving specific cell receptors. More recently, Ghavami and colleagues[191] also provided *in vitro* evidence that S100A8/A9 triggered cell death via a RAGE-independent pathway but involved several components of the mitochondrial death pathway, including Bak, Smac/DIABLO and Omi/HtrA2. Therefore, it seems that the extracellular S100A8/A9 released from phagocytes might be involved in anti-tumour activity, as a host innate immune response against tumour. However, in the present literature, the anti-tumour role of S100A8/A9 has not been confirmed in *in vivo* studies.

On the other hand, Ghavami and colleagues[192] reported tumour-promoting activity of S100A8/A9, in which low concentration of S100A8/A9 promoted cancer cell growth via RAGE signalling and Mitogen-activated protein kinase-dependent pathway. This is further supported by Turovskaya and colleagues[193],

who demonstrated that carboxylated glycans are expressed on a subpopulation of RAGE and mediate S100A8/A9 binding to RAGE, led to activation of NF-ĸB and colonic cancer cell proliferation. In the *in vivo* experiments, the group observed high infiltration of S100A8- and S100A9-expressing myeloid progenitor cells in the stromal regions of dysplasia and adenoma in human colonic tumour tissues and in a mouse model of colitis-associated carcinogenesis. The administration of anti-carboxylated glycan antibody reduces chronic inflammation and tumourigenesis, and that RAGE-deficient mice were resistant to the onset of colitis-associated carcinogenesis. All in all, their findings suggested that inflammation-associated colonic carcinogenesis was mediated by S100A8/A9 through complex tumour-stromal crosstalk. Indeed, Cheng and colleagues[194] also provided *in vivo* evidence that tumour-induced up-regulation of S100A9 in stromal myeloid progenitor cells inhibits dendritic cell and macrophage differentiation, resulted in the accumulation of myeloid-derived suppressor cells (MDSCs) and led to the suppression of anti-tumour immune response. In addition, the MDSCs not only express receptors for S100A8/A9, but also synthesise and secrete these proteins which serve as an autocrine feedback loop that sustains accumulation of MDSCs[195].

More interestingly, recent data has suggested that S100A8/A9 mediates distant tumour metastasis via chemokinetic mechanisms, independent of its direct binding to cancer cells. Hiratsuka and colleagues[196] showed that cytokines such as TNF-α, TGF-β and VEGF were released from tumours, and these factors induced S100A8/A9 in a distant organ. As a chemokine with pro-inflammatory property, the tumour-induced S100A8/A9 in a distant organ plays a role in establishing inflammatory ground that forms pre-metastatic niches, which then

facilitates immigration and metastasis of cancer cells. In a separate study by Hiratsuka and colleagues[197], S100A8/A9 was shown to induce the expression of serum amyloid A3, which binds to toll-like receptor-4 and resulted in NF-ĸB signalling and facilitated metastasis.

1.2.2.3 Smad4 and cancers

Mutations in genes on chromosome 18q21 have been identified as important in colorectal carcinogenesis. Over 60 % of CRC show loss of heterozygosity (LOH) at chromosome 18q[198, 199], in which DCC (Deleted in Colorectal Carcinoma) gene, Smad4 and Smad2 genes have been located[200, 201]. Several studies have shown that LOH at 18q21 in CRC is not just an adverse prognostic factor affecting postoperative survival[202-204], it is also a predictive factor of poor outcome following adjuvant chemotherapy in stage III colon cancer[205]. Evidence from these articles is varied, but loss of heterozygosity or deletion at 18q is generally associated with a reduction in Smad4 protein expression[201, 206].

Smad (Mothers Against Decapentaplegic homologue {Drosophila}) proteins are a group of proteins that modulate the activity of TGF-β ligands. Smads form complexes with other Smads to serve as transcription factors in the cell nucleus. They are divided into three classes[207]:

- The receptor-regulated Smads – Smad1, Smad2, Smad3, Smad5 and Smad9.
- The common-mediator Smads – Smad4 which interact with receptor-regulated Smads in regulating signalling pathways.
- The inhibitory Smads – Smad6 and Smad7 which inhibit the receptor-regulated and common-mediator Smad.

The protein product of the Smad4 gene is a tumour suppressor. It binds to receptor regulated Smads following TGF-β signalling and form the heteromeric complex. This complex migrates into the nucleus initiating gene transcription, which negatively regulates epithelial cell growth[208]. It has been reported that loss of Smad4 occurs in 10-15 % of overall CRC[209], and 30-35 % in tumours with metastases[210]. Howe and colleagues[211] and Woodford-Richens and colleagues[212] demonstrated that germline mutation of the Smad4 gene causes human familial juvenile polyposis. This autosomal dominant disorder is characterised by a predisposition to Smad4-negative gastrointestinal polyps and cancer at a young age. This is further supported by Takaku and colleagues[213, 214] who constructed Smad4 (+/-) knock-out mice that proceeded to develop the phenotype of Familial Juvenile Polyposis, including Smad4-negative gastrointestinal polyps.

Using immunohistochemical assays, Smad4 expression has been investigated in several human cancer types with correlation between Smad4 expression and prognosis reported in pancreatic[215, 216], gastric[217], endometrial[218], breast[219] and oesophagus[220] cancers. Although most studies found that low levels or loss of Smad4 expression correlated with poorer prognosis, Liu and colleagues[218] reported that Smad4 expression was not associated with tumour progression or clinical outcome in endometrial cancer, while Stuelten and collegues[219] observed that patients with Smad4 negative breast cancer tended to have longer survival times compared to Smad4 positive cancer patients.

In CRC, Maitra and colleagues[221] reported that loss of Smad4 in colonic adenocarcinoma correlates with the presence of advanced and metastatic

disease, suggesting that Smad4 inactivation might be associated with the acquisition of a metastatic phenotype. Other studies have reported that Smad4 loss correlated with lymph node[222] and liver metastasis[223]. Alazzouzi and colleagues[224] examined Smad4 protein levels in 86 stage III colorectal cancer specimens and found that low Smad4 levels correlated with poor overall and disease-free survival compared to high levels. Furthermore, Alhopuro and colleagues[225] reported that low Smad4 protein levels were associated with poor prognosis after 5-fluorouracil-based adjuvant therapy.

In the Division of Surgery and Oncology, University of Liverpool, Ms Elizabeth Tweedle employed a tissue microarray-based IHC assay to examine Smad4 expression in 303 stage I-III colorectal cancers and its relationship to clinico-pathological parameters. Smad4 loss was observed in 10.8 % (n = 4/37) of cases in Stage I, 13.5 % (n = 18/133) of Stage II, and 15.6 % (n = 20/128) of Stage III tumour. There were significantly higher cases of Smad4 loss in rectal cancer group (55 %, n = 23/42) than the colon cancer group (45 %, 19/42). Smad4 status was also significantly correlated with pT-stage, with 29 % of cases of pT4 in the Smad4-negative group compared to 11 % in the Smad4-positive group. There was no significant association between Smad4 expression and patients' age, gender, size of tumour, differentiation, resection margin status and pN-stage. Smad4 status was not associated with survival in the node-positive cancer group, but in the node-negative cancer group, patients with Smad4 loss had poorer survival compared to patients with preserved Smad4 expression (Figure 3). Subsequent multivariate Cox regression confirmed that loss of Smad4 expression as an independent prognostic marker for poor survival in patients with node-negative CRC.

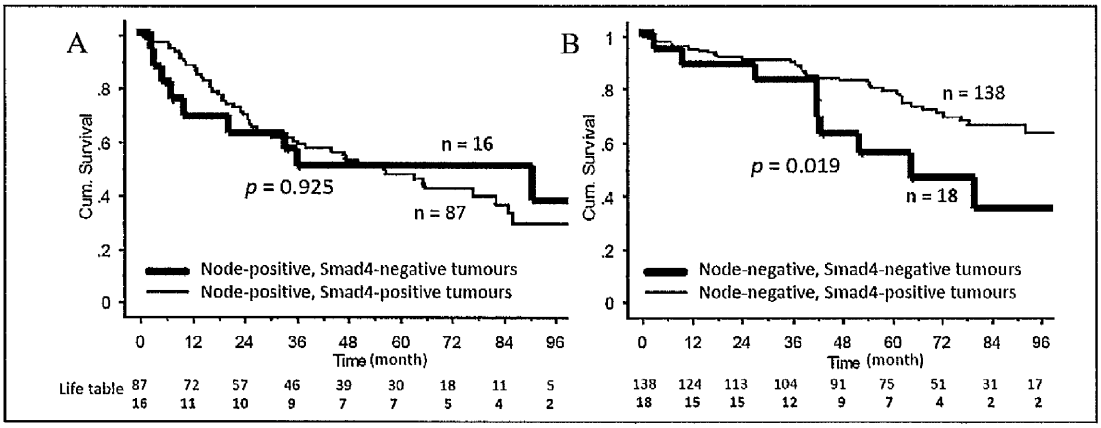


Figure 3: Disease outcome by Smad4 status in colorectal cancer (used with kind permission of Miss Tweedle). (A) In patients with node-positive tumours, no difference in survival was observed according to Smad4 status. **(B)** In patients with node-negative tumours, having Smad4 negative tumours conferred to poorer survival compared to Smad4 positive tumours.

The role of Smad4 inactivation in CRC malignant progression through tumour-stroma crosstalk has been suggested recently. Using a mouse model of colon cancer, Kitamura and colleagues[226] reported that Smad4-negative tumours recruited a specific type of myeloid cell which promoted invasion through crosstalk with tumour cells. Indeed, several proteins have been identified to be differentially secreted by Smad4-deficient colon carcinoma cells which could be relevant for tumour-stroma interactions and carcinogenesis[227].

1.2.3 The heat shock protein 27 (HSP27)

Heat shock proteins (HSPs), also known as stress proteins, are a cohort of highly conserved proteins. Mammalian HSPs are classified into five families

according to their molecular weights (kDa): HSP100, HSP90, HSP70, HSP60, and the small HSPs such as HSP27, HSP40, α-crystallin and HSP17[228]. Each HSP is either constitutively expressed or regulated inductively. For example, in normal cells, HSP70 and HSP27 are either not detectable or expressed at very low levels, whereas HSP90 is abundantly expressed. The expression of HSPs can be induced by a wide range of cellular insults, including heat shock (increased temperature), exposure to chemicals such as ethanol, irradiation, viral infection, and ischaemia-reperfussion phenomena[14]. Several HSPs are also present in the extracellular space, which could be due to the release from necrotic cells or secretion from cells[228]. Evidence has shown that secreted large HSPs such as HSP60 and HSP70 interact with monocytes/macrophages which result in various state of activation, suggesting the roles of extracellular HSPs in cellular response to stress and inflammation[229]. In contrast, extracellular HSP27 has been shown to inhibit the differentiation of monocytes to macrophages or dendritic cells, indicating an immuno-suppressive function of the secreted HSP27[229, 230]. While the roles of intracellular HSP27 in cancer have been largely established and described, its extracellular function has not been linked to cancer.

1.2.3.1 The regulation of HSP27 expression

The expression of small HSPs is controlled at the transcriptional level by the activity of Heat Shock Factors (HSFs) 1-4[231]. Under normal, resting conditions, HSF1 retains its dormant state by binding to HSP70, HSP90, HSP-binding protein 1, or by phosphorylation of HSF-specific proline/serine motifs[232, 233]. Following stress, trimerisation of the HSF1 occurs, and the active trimers then

translocate to the nucleus and bind to the Heat Shock Element located in a specific promoter region of the HSP gene consisting of arrays of the 5-bp unit nGAAn consensus motif arranged as inverted repeats[234]. This induces concomitant expression of both large and small HSPS, which then provides a reciprocal negative feedback for HSF1 activity via HSP70[233]. The induction of HSPs also occurs through a HSF1-independent pathway, namely the oestrogen-sensitive promoter, which accounts for the observed increased expression of small HSPs in oestrogen-sensitive tissues and tumours (Figure 4)[235-237]. The roles of other HSFs are however less well characterised at the present time.

Following transcription of HSPs, post-translational modifications are essential to determine the activity of small HSPs. Small HSPs are phosphorylated at three different sites by mitogen activated protein kinase activated protein kinase 2 (MAPKAPK-2)/stress-activated protein kinase 2 (SAPK-2)/stress-activated kinase p38 (p-38α), MAPKAPK-5, extracellular signal regulated kinases (Erk), G-proteins and protein kinase C[238]. The phosphorylation of HSP27 is mainly regulated by MAPKAPK-2/SAPK-2/p-38α and MAPKAPK-5 pathways. The small HSPs phosphorylated through MAPKAPK-2 pathways play an important role in chemo-resistance and inhibiting apoptosis, whereas MAPKAPK-5 pathway is essential in response to pro-inflammatory cytokines[239, 240].

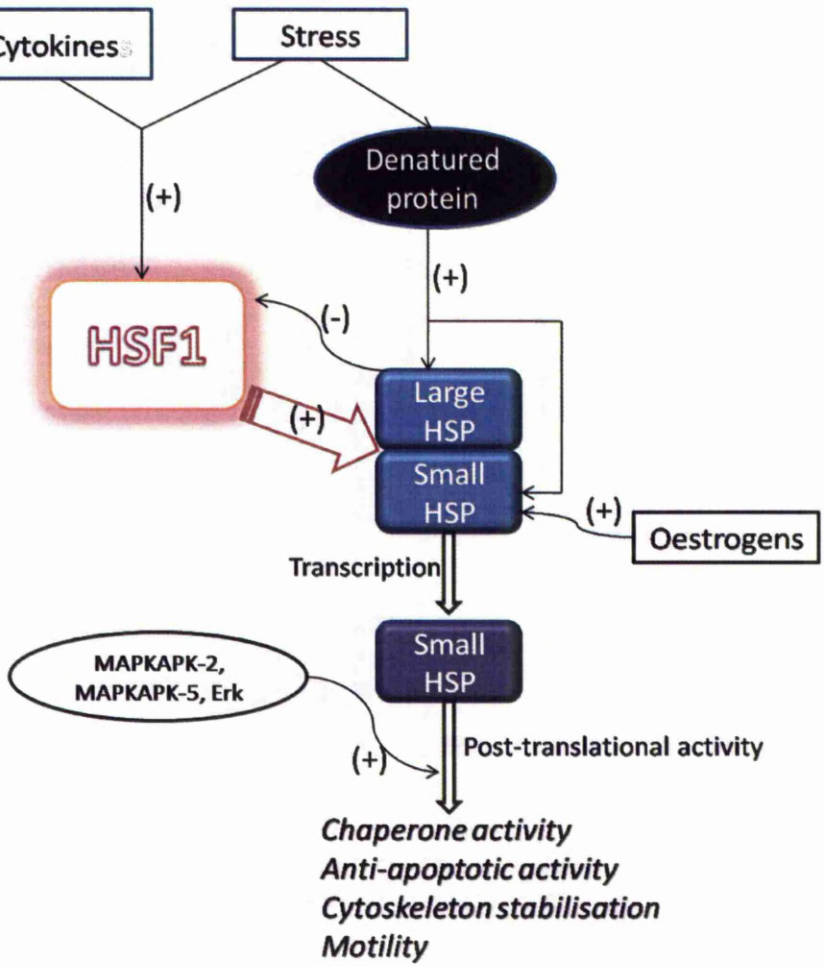


Figure 4: The expression and biological activity of small HSPs are regulated on transcriptional and translational levels[241].

1.2.3.2 The molecular structure of HSP27

The structure of HSP27, like other small HSPs, is characterised by a highly conserved amino acid sequence of 80-100 residues called α-cystallin domain at the C-terminus. These amino acid sequences form β-sheets which are essential for the formation of stable dimers[242]. The C-terminus is flanked by a variable hydrophobic, less conserved region called N-terminus (WD/EPF domain) and a

short flexible C-terminal extension[243]. The flexible C-terminal extension enhances the solubility of small HSPs.

The WD/EPF region at the N-terminus is essential for the development of high molecular oligomers, through aggregation of stable dimers to tetramers and then to unstable oligomers[243]. Cell physiology, the phosphorylation status of HSP27, and the exposure to exogenous stress all dictate HSP27 oligomerisation. Upon phosphorylation, HSP27 multimers dissociate into small oligomers, which are active at conditions where other proteins are denaturing, so they can down-regulate chaperone activity and influence actin polymerisation[244].

1.2.3.3 The functions of intracellular HSP27 and their roles in tumourigenesis

1.2.3.3.1 HSP27 as a molecular chaperone and a potent regulator of cytoskeletal dynamics

The intracellular accumulation of misfolded proteins can induce a stress response and lead to increased HSP expression[245]. These misfolded, damaged proteins can form large aggregates which trigger cellular apoptosis. HSP27 functions as a molecular chaperone by forming complexes with such proteins and by doing so facilitates the repair or destruction of these proteins[246]. HSP27 also stabilises F-actin microfilaments to maintain the structure of the cytoskeleton and prevent its disaggregation during exposures to stress[247]. This ability of HSP27 promotes cell recovery, prevents induction of apoptosis and hence increases cell survival rate. The molecular chaperone function of HSP27 in apoptosis has also been shown in relation to protein kinase B (Akt). During

cellular stress, Akt is activated through an association with HSP27 which then leads to apoptosis inhibition[248]. This HSP27-mediated Akt activation thus confers increased resistance to apoptosis in cells over-expressing HSP27[249].

1.2.3.3.2 The roles of HSP27 in apoptosis pathway

Studies have shown that HSP27 regulates various apoptotic pathways (Figure 5)[250]. HSP27 has been shown to inhibit the activation of pro-caspase-9 by interacting with cytochrome-c[251]. The observation of HSP27 blocking the release of cytochrome-c, suggests that HSP27 may function in a similar manner to another endogenous anti-apoptotic protein, Bcl-2, in preventing the formation of apoptosome complex[252]. In addition, HSP 27 can also regulate apoptosis by inhibiting caspase-3 activity through interaction with pro-caspase-3 molecules.

HSP27 has also been shown to inhibit apoptosis via a cytochrome-c independent pathway, by interacting with Daxx, a nuclear protein that has been implicated as a mediator of Fas-induced apoptosis. Daxx translocates to the cell membrane during Fas-mediated apoptosis, binds to the Fas receptor (also known as death receptor) and also to Ask1, thus mediating a caspase-independent apoptosis[253]. By preventing the translocation of Daxx to the membrane, HSP27 can hinder apoptosis independent of the cytochrome-c and caspase cascade.

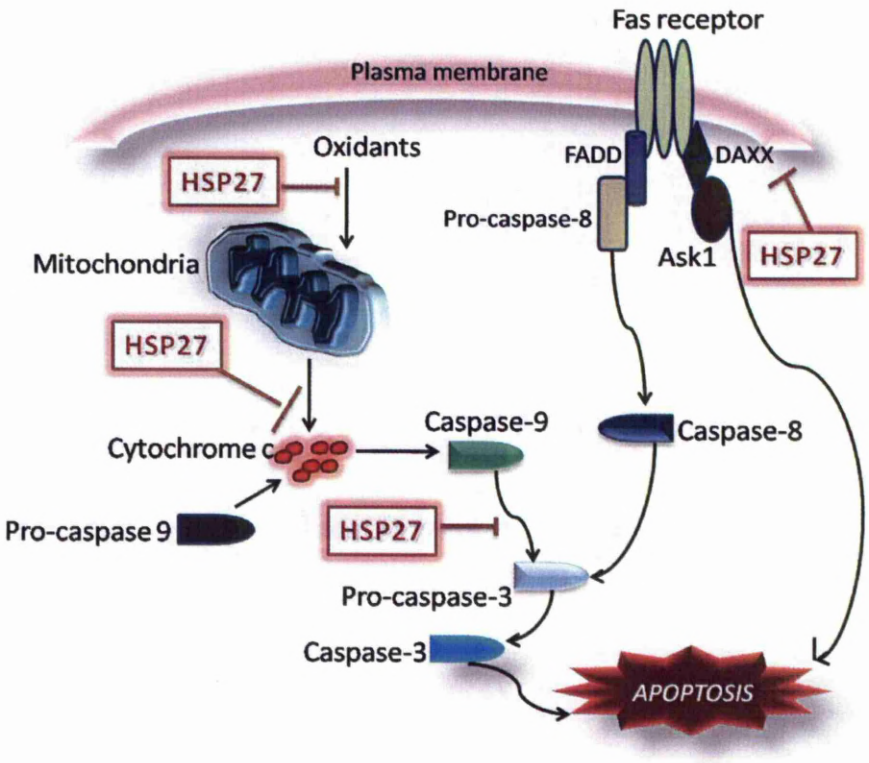


Figure 5: HSP27 inhibits apoptosis via several pathways[250].

By understanding the complex roles of HSP27 in cellular homeostasis and apoptosis, it is not surprising that the aberrant expression of HSP27 is associated with tumourigenesis. Inappropriately high level of HSP27 can result in dysregulation of apoptotic signalling which is a common feature in cancer initiation, growth and progression. As such, the protective role of HSP27 to cellular insults may also render tumours resistant to chemotherapeutic agents.

1.2.3.4 The expression of HSP27 in human cancers and its clinical repercussions

The expression of HSP27 has been reported in a wide range of human cancers. Numerous studies have provided evidence on the expression of HSP27 in

tumours and its repercussions in the clinical setting, particularly in cancer diagnosis, prognosis, prediction and treatment.

The differential expression of HSP27 has been implicated in several hormone-dependent malignancies such as breast, endometrial, ovarian and prostate cancer. In breast cancer, the immune-cytochemical staining of fine needle aspiration smears for HSP27 positively predicts the presence of malignancy[254]. High expression of HSP27 has also been detected in the serum of patients with breast cancer using two-dimensional electrophoresis (2-DE) followed by mass spectrometry (MS) identification[255]. However, very conflicting data exist about the relationships between HSP27 expression, metastasis and survival in breast carcinoma. For example, the expression of HSP27 in breast cancer is associated with small tumour size and low proliferative index, but its expression in node negative cases is linked with short disease-free survival[256]. Several studies have also shown that the expression of HSP27 in breast cancer has no correlation with disease-free survival or overall survival[257-259]. In ovarian carcinoma, high HSP27 expression is associated with more advanced tumours[260]. HSP27 is also associated with increased hyperplastic endometrium[261], positively correlated with oestrogen receptor[262], and overexpressed in some endometrial malignancy[236]. In prostate cancer, Storm and colleagues[263] reported that HSP27 is absent in the normal and malignant tissues. However, Conford and colleagues[264] found that the early in-situ neoplastic transformation of normal prostatic epithelium is associated with loss of HSP27 expression, and that in advanced cancers, HSP27 expression is associated with poor clinical outcome.

The clinical implication of HSP27 expression has also been reported in other cancer types. In osteosarcoma[265] and gastric[266, 267] cancer, HSP27 expression is associated with poor overall survival. In leukaemia[268], oral[269], renal[270] and bladder[263] cancers, HSP27 expression has no correlation with disease-free survival or overall survival. Interestingly, lower expression of HSP27 is associated with poor survival in squamous cell oesophageal carcinoma[271] and high expression of HSP27 is correlated with better prognosis[272].

Several studies have also explored the use of HSP27 expression to predict the response of cancer patients to specific treatments (Table 7). Given the cyto-protective role of HSP27 in apoptosis, it is not surprising that the role of HSP27 in chemoresistance has been largely studied and relationship to outcome determined in many cancer types. For cancers such as ovarian[260], oesophageal[273], prostate[274] and pancreas[275] carcinomas, high levels of HSP27 are associated with chemo or radio-resistance and poor prognosis. These studies provide proof-of-principle that HSP27 can potentially be used to tailor the treatment strategy for individual cancer patients. However, findings remain inconclusive in breast cancer, with high HSP27 linked to poor response to chemo- and endocrine therapy in a study[276], with no association with therapy detected in others[257, 277].

Cancer types	Study	n	Findings
Breast	Seymour *et al.*, 1990[276].	51	HSP27 expression correlated with better response to chemotherapy and tamoxifen for Oestrogen Receptor+ tumours.
	Damstrup *et al.*, 1992[257].	119	Did not predict response to endocrine therapy.
	Ciocca *et al.*,	205	Did not predict response to tamoxifen.

	1998[277].		
Ovarian	Langdon *et al.*, 1995[260].	72	High expression correlated with resistant to chemotherapy.
Oesophageal	Takeno *et al.*, 2001[273].	34	HSP27 predicted the therapeutic effect of combined radiotherapy and chemotherapy in T4 oesophageal squamous cell carcinoma.
Rectal	Rau *et al.*, 1999[278].	23	Differential HSP27 expression did not correlate with the level of clinical response in patients treated with pre-operative chemotherapy, radiotherapy or hyperthermia for locally advanced rectal cancer.
Prostate	Bubendorf *et al.*, 1999[274].	264	Overexpression of HSP27 correlated with 31 % of hormone refractory tumours, 5 % of primary tumours and 0 % in benign prostate specimens.
Pancreas	Mori-Iwamoto *et al.*, 2007[275].	11	Increased HSP27 expression in tumour specimens was related to higher resistibility to gemcitabine in patients of pancreatic cancer.
Colorectal	Choi *et al.*, 2007[279].	20	HSP27 expression levels of tumour specimens from irinotecan-non-responding patients were significantly higher compared to those of irinotecan-responding patients.

Table 7: The predictive implications of HSP27 in various cancer types.

The association of HSP27 in several cancer types and clinical correlations has generated an exciting new avenue in the field of cancer treatment via two main strategies: pharmacological modifications of HSP expression or molecular chaperone activity, and the use of HSP as adjuvant to present tumour antigens to the immune system[280]. At present, the study of cancer treatment by targeting

HSP27 is still in its infancy at the molecular level in cell lines and animal experiments, utilising short interfering RNA (siRNA) or antisense oligonucleotide (ASO) to suppress protein expression. Several such studies have been reported with promising outcomes (Table 8).

Authors	Findings
Rocchi *et al.*, 2004[281].	Antisense oligonucleotide (ASO)-induced silencing of HSP27 in a prostate cancer cell line enhanced apoptosis and delayed tumour progression.
Yang *et al.*, 2008[282].	The suppression of HSP27 expression by HSP27 ASOs enhanced vincristine and adriamycin chemosensitivity in a human gastric cancer cell line.
Kamada *et al.*, 2007[283].	HSP27 ASO inhibited bladder tumour growth and enhanced paclitaxel sensitivity in mice.
Rocchi *et al.*, 2005[284].	HSP27 ASO treatment in athymic mice bearing prostate tumours significantly delayed tumour growth after castration, decreasing mean tumour volume and serum prostate-specific antigen levels by 57 % and 69 % respectively.
Rocchi *et al.*, 2006[285].	Silencing of HSP27 in prostate cancer cells by siRNA increased apoptotic rates 2.4-4 fold and caused 40-76 % inhibition of cell growth in prostate cancer cells.
Zhang *et al.*, 2007[286].	Knockdown of HSP27 resulted in an increase of the apoptotic fraction from ~22 % to 31 % in the cervical cancer cells exposed to cisplatin chemotherapy.
Zoubeidi *et al.*, 2007[287].	Knockdown of HSP27 using the antisense drug OGX-427 increased prostate cancer cell apoptotic rates.
Hadaschik *et al.*, 2008[288].	Intravesical OGX-427 (HSP27 ASO) instillation therapy targeting HSP27 showed promising anti-tumour activity and minimal toxicity in an orthotopic mouse model of high-grade bladder cancer. These findings provide pre-clinical proof-of-principle for the use of ASO as an intravesical agent for non-muscle-invasive bladder cancer.

Mori-Iwamoto *et al.*, 2007[275].	Knocked down HSP27 in a gemcitabine-resistant pancreatic cancer cell line resulted in restoration of gemcitabine sensitivity.
Choi *et al.*, 2007[279].	Inhibition of HSP27 expression in several human colorectal cancer cell lines using an ASO increased irinotecan sensitivity in cancer cells.
Aloy *et al.*, 2008[289].	HSP27 protects against gamma irradiation-induced apoptosis in several cancer cell lines. Inhibition of HSP27 expression radiosensitised these radioresistant cells, suggesting that targeting HSP27 offers a potential adjuvant to radiation-based therapy of resistant tumours.
Garrido *et al.*, 1996[290].	Colonic cancer cells were stably transfected with HSP27 anti-sense or sense. Cells over-expressed HSP27 showed increased resistance to doxorubicin as compared to increased sensitivity to doxorubicin among cells under-expressed HSP27. The sensitivity of the different transfected cells to 5-FU was no difference.

Table 8: Targeting HSP27 in cancer treatment (*in vitro* and in *vivo* studies).

The evidence of clinical implications of HSP27 in colorectal cancer is relatively sparse in the present literature. Rau and colleagues[278] reported that in patients with locally advanced rectal cancer, HSP27 was detectable in most tumours and surrounding tissues before and after neo-adjuvant treatment. However, the differential HSP27 expression did not correlate to the level of clinical response in patients treated with pre-operative chemotherapy, radiotherapy or hyperthermia. This study involved only 23 patients. In addition, Choi and colleagues[279] examined the expression of HSP27 in the surgically resected primary colorectal tumours from 20 patients using immunohistochemistry. The level of HSP27 expression was then correlated to

patients' clinical response to adjuvant irinotecan therapy. The group found that among patients who did not response to irinotecan, the level of HSP27 expression in the tumours was significantly higher compared to those of irinotecan-responder, suggesting that HSP27 is involved in the irinotecan resistance of CRC. The group also reported that HSP27 inhibition using ASO increased the irinotecan sensitivity of colorectal cancer cells.

Using a proteomic approach similar to that described above (section 1.2.2.2.3, page 37) for the identification of differential expression of S100A8 in the stroma of colorectal tumours, Mr Ilyas Khattak and Dr Taoufik Nedjadi identified differential expression of HSP27 protein in colorectal tumours (see Figure 1 of the published paper, Tweedle EM, Khattak I, Ang CW and colleagues[138] appended to this thesis).

To validate the proteomic findings and to establish the cellular basis for the variability of HSP27, immunohistochemistry (IHC), for the detection of HSP27 in 404 formalin-fixed colorectal adenocarcinomas was undertaken by Ms Elizabeth Tweedle. While the variability in HSP27 expression was not altered by pre-operative administration of radiotherapy and largely no association with patient clinico-pathological parameters was observed, high HSP27 expression was associated with incomplete resection margins in rectal cancer patients. More importantly, high HSP27 expression was strongly correlated with poor cancer-specific survival in rectal cancer but not colonic cancer (see Figure 3 of Tweedle and colleagues[138]). Further multivariate statistical analyses showed that high HSP27 expression was an independent predictor of survival in rectal cancer patients.

CHAPTER 2:
AIMS & OBJECTIVES

As described in the Introduction, in my project I undertook analysis of aspects of S100A8/A9 and HSP27 in colorectal cancer. The Aims and Objectives of these distinct pieces of research are set out separately below in sections 2.1 and 2.2 respectively.

2.1 The roles of S100A8 and S100A9 and their relationship to tumoural Smad4 in carcinogenesis.

The expression of S100A8 and S100A9 proteins has been reported in several cancer types and the proteins have been implicated in carcinogenesis via crosstalking with cancer cells. Recent work in the Division of Surgery and Oncology, University of Liverpool has shown that S100A8 and S100A9 were localised to colorectal tumour-associated inflammatory cells and that the microenvironment of Smad4-negative colorectal tumours had significantly reduced numbers of S100A8-expressing cells compared to the microenvironments of Smad4-positive tumours. We therefore wondered whether the expression of Smad4 in cancer cells affected their response to exogenous S100A8 and S100A9. The aims and objectives of this research were:

Aim 2.1.1: To examine the effects of S100A8 and S100A9 on colorectal cancer cell motility and proliferation.

Aim 2.1.2: To determine if depleting Smad4 in colorectal cancer cells would influence the impact of S100A8 and S100A9 on cell motility and proliferation.

Aim 2.1.3: To determine the relationship between S100A8 and S100A9 intracellular signalling in relation to Smad4 and the RAGE receptor.

Objective 2.1.1: To generate recombinant S100A8 and S100A9 proteins.

Objective 2.1.2: To carry out motility (modified Boyden Chamber) and proliferation (MTS) assays in order to determine cell motility and proliferation in the presence of exogenous recombinant S100A8 and S100A9 proteins.

Objective 2.1.3: To characterise the expression of Smad4 in colorectal cancer cell lines and to deplete (knockdown) Smad4 in these cells.

Objective 2.1.4: To carry out motility and proliferation assays on Smad4 depleted cells in the presence of S100A8 and S100A9 proteins.

Objective 2.1.5: To characterise the effect of exogenous S100A8 and S100A9 on the cellular expression of phospho-Smad2, phospho-Smad3 and phospho-Smad1/5/8 in the presence/absence of anti-RAGE receptor antibody using Western blotting and cyto-immunofluorescence.

The outcome of these experiments could help to determine some of the potential functional consequences of high levels of S100A8 and S100 A9 in the microenvironment of CRC tumours and unravel the relationship between S100A8, S100A9 and Smad4 in CRC.

2.2 The clinical relevance of tumoural HSP27 expression as a predictive marker of response to adjuvant therapy in colorectal cancer

The current literature suggests that HSP27 overexpression correlates with poor clinical prognosis in several cancer types and that HSP27 depletion in cancer cells increases chemotherapy efficacy *in vitro*. Recent work in the Division of Surgery and Oncology, University of Liverpool indicated that HSP27 overexpression correlated with poor survival in patients with rectal but not colonic cancer. However, a comprehensive analysis of the effects of HSP27 levels on treatment response in these patients was lacking.

It has been reported in the literature that HSP27 depletion in colonic cell lines enhanced cell sensitivity to irinotecan and doxorubicin. To our knowledge, nothing is known about the effects of HSP27 levels on the response of rectal cancer cells to treatment. The aims and objectives of this research were:

Aim 2.2.1: To examine if HSP27 expression was predictive of response to neoadjuvant/adjuvant therapy in CRC patients.

Aim 2.2.2: To examine colorectal cancer cell behaviour upon HSP27 depletion, with a particular focus on rectal cancer cells, which have received little attention in the past.

Aim 2.2.3: To determine if depleting HSP27 expression would increase the effectiveness of chemotherapy and radiotherapy in killing or slowing the growth of colorectal cancer cells.

Objective 2.2.1: To obtain, for a cohort of 404 CRC patients for which a large database of clinico-pathological data and HSP27 expression already existed, 1. Detailed neoadjuvant and adjuvant therapy data from the Clatterbridge Centre

for Oncology, Wirral, UK and 2. Verified causes of death from the North West Cancer Intelligence Service, Liverpool, UK.

Objective 2.2.2: To use the information obtained in Objective 2.1.1 to determine whether HSP27 expression was predictive of response to neoadjuvant/adjuvant therapy in CRC patients.

Objective 2.2.3: To characterise the expression of HSP27 in colon and rectal cancer cell lines and to deplete these levels by siRNA-mediated knockdown.

Objective 2.2.4: To determine the IC_{50} of three chemotherapeutic agents: 5-fluorouracil, oxaliplatin and irinotecan and radiotherapy for each cell line, including rectal cell lines where no published data were available.

Objective 2.2.5: To carry out apoptosis assays following incubation of HSP27 depleted cells with chemotherapeutic agents or exposure to radiotherapy.

Objective 2.2.6: To examine cell cycle profiles, cell motility (modified Boyden Chamber) and proliferation (MTS assay) on control and HSP27-depleted cells.

The outcomes of analyses on the large cohort of CRC patients could lead to a greater understanding on the predictive role of HSP27 in response to adjuvant treatment. The *in vitro* experiments would inform whether targeting HSP27 in colon and rectal cancer cells could enhance the efficacy of chemo/radiotherapy. This would have a major impact on new cancer drug development and future direction in neo-adjuvant/adjuvant therapy of colorectal cancer.

CHAPTER 3:
MATERIALS & METHODS

3.1 Materials.

The reagents used in this study are summarised in Table 9 below.

Materials	Company	Product code
Acrylamide-bis ready-to-use solution 30% for electrophoresis	VWR international, Lutterworth, Leics, UK	1.00639.1000
Ammonium persulfate	Sigma Aldrich, Gillingham, Dorset, UK	A3678
Ampicillin sodium salt	Sigma Aldrich, Gillingham, Dorset, UK	A9518
Annexin V-FITC Apoptosis Detection Kit	Abcam, Cambridge, UK	ab14085
Aqua-resist 100 ml	VWR international, Lutterworth, Leics, UK	462-7000
Bacto™ Tryptone, Enzymatic digest of casein	BD, Oxford, UK	211705
Bacto™ Yeast Extract	BD, Oxford, UK	212750
bisBenzimide H (Hoechst stain)	Sigma Aldrich, Gillingham, Dorset, UK	B2883
Blotting paper Whatman GB004	VWR international, Lutterworth, Leics, UK	732-4234
BCA Protein Assay Kit	ThermoFisher Scientific, Surrey, UK	23225
BD cell insert with 8.0 micron membrane for 24 well plate	Scientific Laboratory Supplies, Nottingham, UK	353097
β-Mercaptoethanol	Sigma Aldrich, Gillingham, Dorset, UK	M6250
Bovine serum albumin solution	Sigma Aldrich, Gillingham, Dorset, UK	A7284
Bromophenol Blue	Sigma Aldrich, Gillingham, Dorset, UK	B0126
Centrifuge Tube 15mL, Conical, Loose, Sterile	Star Labs, Milton Keynes, UK	E1415-0200
Centrifuge Tube 50mL, Conical, Loose, Sterile	Star Labs, Milton Keynes, UK	E1450-0200
Citric acid monohydrate	Sigma Aldrich, Gillingham, Dorset, UK	C7129
Coomassie Blue	Sigma Aldrich, Gillingham, Dorset, UK	C4351
Cuvet Semi Micro PS 1 * 100 items)	VWR international, Lutterworth, Leics, UK	634-2501
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Gillingham, Dorset, UK	D5879
DL-Dithiothreitol	Sigma Aldrich, Gillingham, Dorset, UK	D9779
DPX mountant for microscopy	VWR international, Lutterworth, Leics, UK	360292F
Dulbecco's Modified Eagle's Medium	PAA, Pasching, Austria	E15-810
Eight-Well Culture Slide	VWR international, Lutterworth, Leics, UK	734-0089
EZ4U - Non-radioactive cell proliferation and cytotoxicity assay	Biomedica, Vienna, Austria	BI-5000
Foetal Bovine Serum Gold	PAA, Pasching, Austria	A15-151
Geneticin (G418) Sulphate	GIBCO, Paisley, UK	345812

Glycerin	Sigma Aldrich, Gillingham, Dorset, UK	G2289
Glycerol	BDH Laboratory Supplies, Dorset, UK	101184K
Glycine	ThermoFisher Scientific, Surrey, UK	BP381-5
Goat anti-human RAGE antibody	R&D Systems, Abingdon, UK	AF1145
Goat anti mouse immunoglobulins HRP	Dako, Ely, Cambridgeshire, UK	P0447
Goat anti-rabbit IgG antibody (fluorescein isothiocyanate-conjugated)	Jackson ImmunoResearch Laboratories Inc., PA, USA	GT26001-1000
Goat anti rabbit immunoglobulins HRP	Dako, Ely, Cambridgeshire, UK	P0448
Goat serum	Sigma Aldrich, Gillingham, Dorset, UK	G9023
Glutathione, reduced	VWR international, Lutterworth, Leics, UK	1.04090.0005
Glutathione Sepharose	GE Healthcare, Amersham, Bucks, UK	17-0756-01
Hanks' balanced salt solution	Sigma Aldrich, Gillingham, Dorset, UK	H6648
HEPES	Sigma Aldrich, Gillingham, Dorset, UK	H3375
Human HSP27-targetting SiRNA1 siGENOME	Dharmacon Inc., Chicago, US	D-005269-03
Human HSP27-targetting SiRNA2 siGENOME	Dharmacon Inc., Chicago, US	D-005269-05
Human insulin solution	Sigma Aldrich, Gillingham, Dorset, UK	I9278
Human non-targeting siRNA control1	Dharmacon Inc., Chicago, US	BOYMA-000021
Human non-targeting siRNA control2	Dharmacon Inc., Chicago, US	D-001210-0X
Human Smad4-targetting siRNA1 siGenome	Dharmacon Inc., Chicago, US	D-003902-05
Human Smad4-targetting siRNA2 siGenome	Dharmacon Inc., Chicago, US	D-003902-06
HRT-18	European Collection of Cell Cultures (ECACC), Salisbury, UK	86040306
Hydrochloric acid	Sigma Aldrich, Gillingham, Dorset, UK	H0636
Irinotecan	Sigma Aldrich, Gillingham, Dorset, UK	I1406
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Calbiochem, Darmstadt, Germany	420291
L-15 Medium (Leibovitz)	Sigma Aldrich, Gillingham, Dorset, UK	L5520
Lipofectamine 2000	Invitrogen, Renfrew, UK	11668-019
Microcentrifuge tubes, safe-lock 0.5ml	VWR international, Lutterworth, Leics, UK	211-2140
Microcentrifuge tubes, safe lock. 2.0 ml	VWR international, Lutterworth, Leics, UK	211-2120
Mouse anti-β-Actin antibody	Sigma Aldrich, Gillingham, Dorset, UK	A5441
Mouse anti-human HSP27 antibody	Santa Cruz, Heidelberg,	sc13132

	Germany	
Mouse anti-human SMAD4 antibody	Santa Cruz, Heidelberg, Germany	sc7966
Mouse anti-RAGE antibody	Santa Cruz, Heidelberg, Germany	sc80652
Mowiol	VWR international, Lutterworth, Leics, UK	475904-100
Mycoplasma testing control (Positive and negative) slides	Bionique Testing Laboratories, NY, USA	M-600
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma Aldrich, Gillingham, Dorset, UK	T7024
Nitrocellulose membrane Hybond ECL 30cm*3m	GE Healthcare, Amersham, Bucks, UK	RPN303D
Non-fat dry milk, Blotting-Grade Blocker	Biorad, Hertfordshire, UK	170-6404
OPTIMEM® I Reduced Serum Medium (1X), liquid - with L-Glutamine	GIBCO, Paisley, UK	31985-047
Oxaliplatin	Sigma Aldrich, Gillingham, Dorset, UK	O9512
PageRuler Prestained Protein Ladder	Fermentas, York, UK	#SM0671
Paraformaldehyde	BDH Laboratory Supplies, Dorset, UK	28794.295
Phosphatase Inhibitor Cocktail Tablets supplied in EASYpacks	Roche Applied Sciences, Burgess Hill, UK	04906845001
Phosphate Buffered Saline tablets (Dulbecco `A' - 50 Tablets)	Oxoid, Hampshire, UK	BR0014G
Poly-Prep Chromatography Columns	Biorad, Herdfordshire, UK	731-1550
Protease Inhibitor cocktail tablets	Roche Applied Sciences, Burgess Hill, UK	11836170001
Quick-Diff Stain kit	IBG IMMUCOR, West Sussex, UK	102164
Rabbit polyclonal anti-GAPDH (FL-335) Antibody	Santa Cruz, Heidelberg, Germany	Sc-25778
Rabbit anti-phospho-Smad1/Smad5/Smad8 antibody	Cell Signalling Technology, Massachusetts, USA	9511
Rabbit anti-phospho-Smad2 antibody	Cell Signalling Technology, Massachusetts, USA	3108
Rabbit anti-phospho-Smad3 antibody	Cell Signalling Technology, Massachusetts, USA	9520
Rabbit anti-phospho-Smad2/Smad3 antibody	Cell Signalling Technology, Massachusetts, USA	9510
Rhodamine phalloidin	Invitrogen, Renfrew, UK	R415
Ribonuclease A from bovine pancreas.	Sigma Aldrich, Gillingham, Dorset, UK	R5250
RNAse free water	Dharmacon Inc., Chicago, US	B-003000-WB-045
RNase inhibitor	Promega, Southampton, UK	N2511
RPMI-1640 Medium	PAA, Pasching, Austria	E15-840
Sodium chloride	Sigma Aldrich, Gillingham, Dorset, UK	S7653
Sodium dodecyl sulfate SigmaUltra	Sigma Aldrich, Gillingham, Dorset, UK	L6026
Sodium Hydroxide	Sigma Aldrich, Gillingham, Dorset, UK	S8045

Sodium phosphate dibasic	Sigma Aldrich, Gillingham, Dorset, UK	S7907
Staurosporine	Sigma Aldrich, Gillingham, Dorset, UK	S4400
SW 837, Human Caucasian rectum adenocarcinoma	European Collection of Cell Cultures (ECACC), Salisbury, UK	91031104
T25 cell culture flask	Nunc, NY, USA	156367
T75 cell culture flask	Nunc, NY, USA	156499
TGF-β	PeproTech EC Ltd, London, UK	100-21C
Tris (hydroxymethyl)methylamine	BDH Laboratory Supplies, Dorset, UK	443866G
Triton® X-100	Sigma Aldrich, Gillingham, Dorset, UK	T8532
Trypsin-EDTA solution	Sigma Aldrich, Gillingham, Dorset, UK	T3924
TWEEN® 20	Sigma Aldrich, Gillingham, Dorset, UK	P1379
Western Lighting Chemiluminescence Reagent Plus	PerkinElmer Life Sciences, Inc, Cambridge, UK	NEL105001EA
X-Ray Kodak Film 18x24 cm	ThermoFisher Scientific, Surrey, UK	AUT-300-040D

Table 9: Materials.

3.2 Methods

3.2.1 Cell lines and cell culture

Four colonic (SW480, HCT116, SWK3 and SWD20), two rectal (HRT18 and SW837) and one pancreatic cancer cell line, PANC1 (used as a control, since the research group in which I worked had much relevant data with this cell line) were initially cultured and maintained as outline in Table 10. The SW480, HCT116 and PANC1 cell lines were from the liquid nitrogen storage of the Division of Surgery and Oncology, School of Cancer Studies, University of Liverpool. The SW837 and HRT18 were purchased from the European Collection of Cell Cultures. The SWK3 and SWD20 were kind gifts from Dr Irmgard Schwarte-Waldhoff (University of Bochum, Germany), and were

described previously[291, 292]. Briefly, they are neomycin resistant clonal derivatives of a Smad4-deficient SW480 cells, stably re-expressing Smad4 (SWD20) and a negative control transfectant (SWK3), and were maintained in medium containing geneticin (0.2 mg/mL). All these cell lines display adherent growth and an epithelial morphology. They were last authenticated by Mr Robert Ferguson (laboratory technician of the Division of Surgery and Oncology, University of Liverpool) in October 2009 using short tandem repeat profiling against the international reference standard for cell lines.

All cell culturing media were supplemented with 10 % Foetal Bovine Serum (FBS), L-glutamine 5 ml (2 nM), and penicillin-streptomycin solution 5 mL. All media, phosphate buffered saline and trypsin were warmed to 37 ^{0}C in a waterbath prior to use.

Culturing of cells was undertaken using aseptic technique in a Class II laminar flow tissue culture cabinet. Cells were cultured as monolayer at 37 ^{0}C with 5 % CO_2 except for SW837 cells, which were maintained in a CO_2-free incubator. Cells were routinely maintained in 75 cm^2 tissue culture flasks and sub-cultured every 3-4 days, when confluency reached approximately 80-90 %.

For subculturing, culture medium was removed and cells were washed with 10 mL sterile Dulbecco Phosphate Buffered Saline (PBS) to remove any residual medium. 2 mL of Trypsin-EDTA solution (0.5 g porcine trypsin and 0.2 g EDTA in Hanks' Balanced Salt Solution with Phenol Red) were added to cover the cells and then incubated at 37 ^{0}C for several minutes until the cells detached from the flask base. The trypsin was then neutralised with 8 mL of culture medium. Cells were repeatedly aspirated and released from a sterile pipette to

produce a single cell suspension, and then re-plated at the ratios shown in Table 10. Fresh culture medium was then added to the flasks to a total of 20 mL.

Cell line	Organ source	Tumour Stage	Disease	Culture medium	Splitting ratio
SW480 (and subclones SWD20 and SWK3)	Colon	Stage II	Adenocarcinoma	Dulbecco Modified Eagle's Medium	1:10
SW837	Rectum	Stage IV	Adenocarcinoma	L-15 Leibovitz's medium	1:5
HCT116	Colon	Stage III	Adenocarcinoma	Dulbecco Modified Eagle's Medium	1:10
HRT18	Rectum	Stage III	Adenocarcinoma	RPMI-1640 medium	1:10
PANC1	Pancreas	-	Adenocarcinoma	RPMI-1640 medium	1:10

Table 10: Cell lines used in the experiments.

3.2.2 Cell freezing and thawing

Cell freezing media	55 % of serum free medium, 25 % of Foetal Bovine Serum, 20 % of DMSO

Box 1: Reagents used in cell freezing for storage in liquid nitrogen.

For cell freezing, cells were harvested following trypsinisation as described above and subjected to centrifugation at 1300 rpm for 8 minutes. The pellet was then resuspended in 250 µL of freezing medium (Box 1) diluted with 750 µL of normal medium, and aliquoted into sterile cryo-vials. The vials were kept on ice for 1 hour, followed by storage at -80 ^{0}C for 48 hours before transferring to liquid nitrogen.

For cell thawing from liquid nitrogen storage, cells were thawed at 37 ^{0}C and immediately transferred into a 15 mL tube containing pre-warmed PBS. Following centrifugation at 1300 rpm for 8 minutes, this washing step was repeated once more before transferring cells to culture medium.

3.2.3 Mycoplasma detection using DNA staining

Citrate-Phosphate working buffer	Solution I: 28.39 g of dibasic sodium phosphate dissolved in 1 L of distilled water. Solution II: 10.51 g of citric acid monohydrate and 14.2 g of dibasic sodium phosphate dissolved in 1 L of distilled water. Small amount of Solution I added to 1 L of Solution II until a pH of 5.5, autoclaved and stored at 4 ^{0}C.
Glycerin mounting medium	Equal volume of glycerin was mixed with citrate-phosphate working buffer, filtered and stored at 4 ^{0}C.
Stock DNA stain	5 mg of bisBenzimide H (Hoechst stain) added to 100 mL of Hanks' balanced salt solution, mixed for 30 minutes while protected from light, filtered and stored at 4 ^{0}C.
Working DNA stain	0.3 mL of stock DNA stain made up to 100 mL with citrate-phosphate working buffer protected from light.
Carnoy's Fixative	3 parts methanol: 1 part acetic acid.

Box 2: Reagents used in the experiment for mycoplasma detection.

Using a 24-well plate and 1cm diameter glass slide/well, 5 x 10^{4} cells/mL (duplicate) were plated, incubated overnight with 1mL/well of antibiotic-free cell culture media in each well.

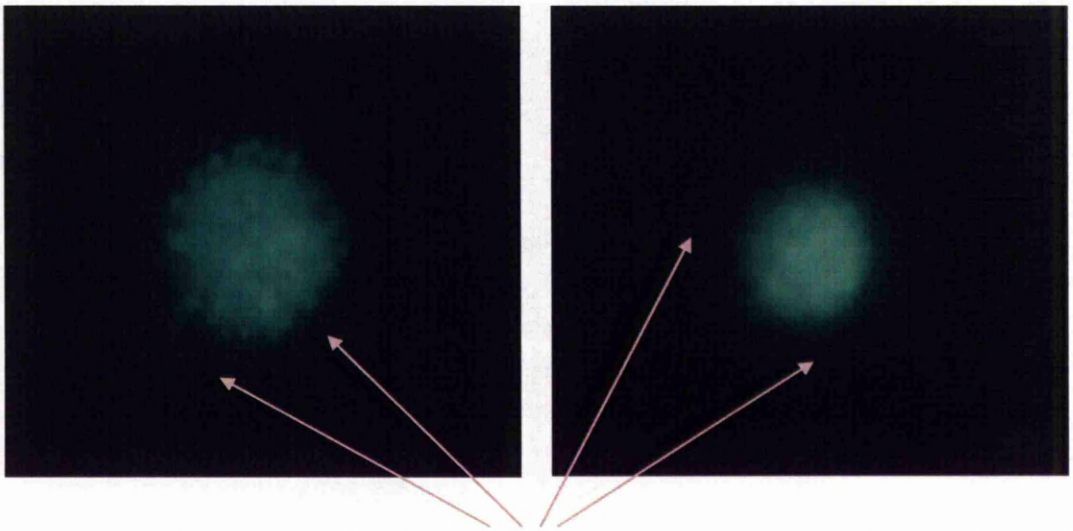
The following day, cell culture media were discarded, and 1 mL of Carnoys' fixative (Box 2) was added and incubated for 5 minutes. Then, the fixative was

discarded; another 1 mL fixative was added and incubated for 10 minutes. The glass slide was then left to air dry for at least an hour.

The air-dried slides, along with the prefixed control (positive and negative control) slides were immersed into a staining chamber containing enough working DNA stain (Box 2) to fully cover the slide. This was incubated at room temperature in the dark for 30 minutes.

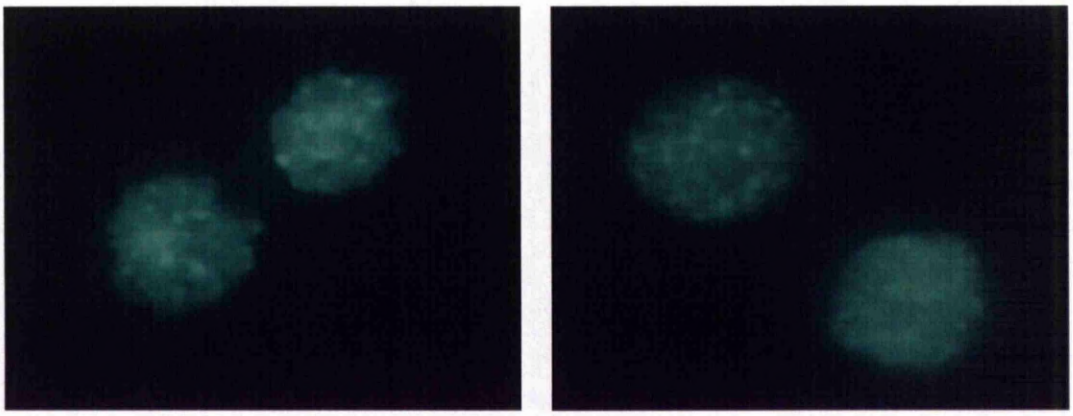
Then, the staining solution was discarded, the slides were rinsed with distilled water twice, and mounted on a microscopic slide with drops of glycerine mounting medium (Box 2). This was then covered with a large cover glass, and viewed under a fluorescent microscope (Figure 6).

Positive controls:



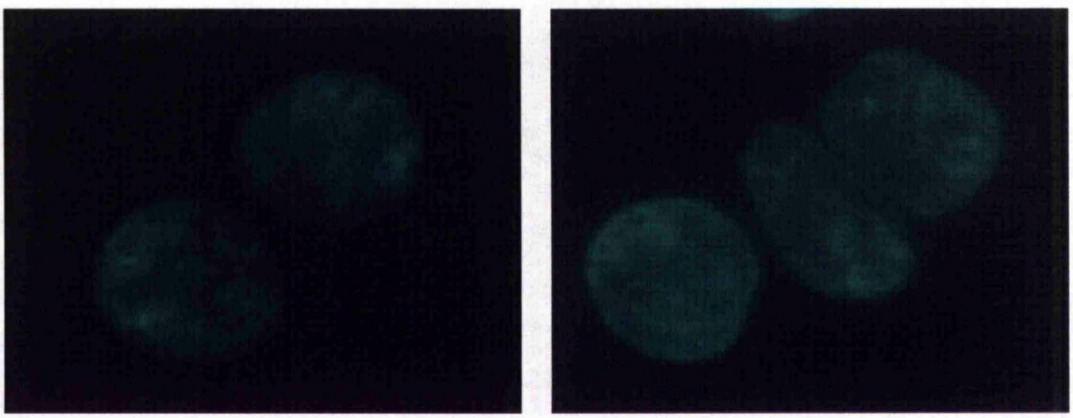
The presence of mycoplasma DNA fluorochrome staining in the cytoplasm confirms mycoplasma infected cells.

Negative controls:

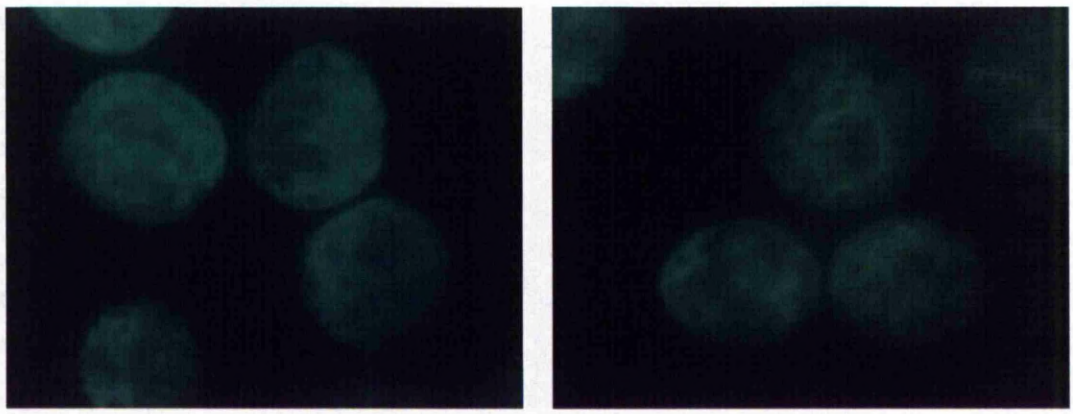


The absence of mycoplasma DNA fluorochrome staining in the cytoplasm confirms mycoplasma-free cells.

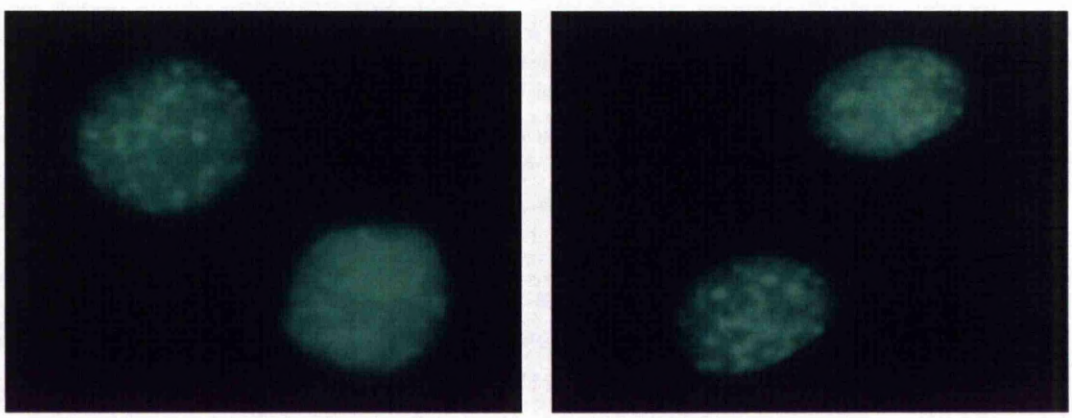
SW480 - mycoplasma-free cells



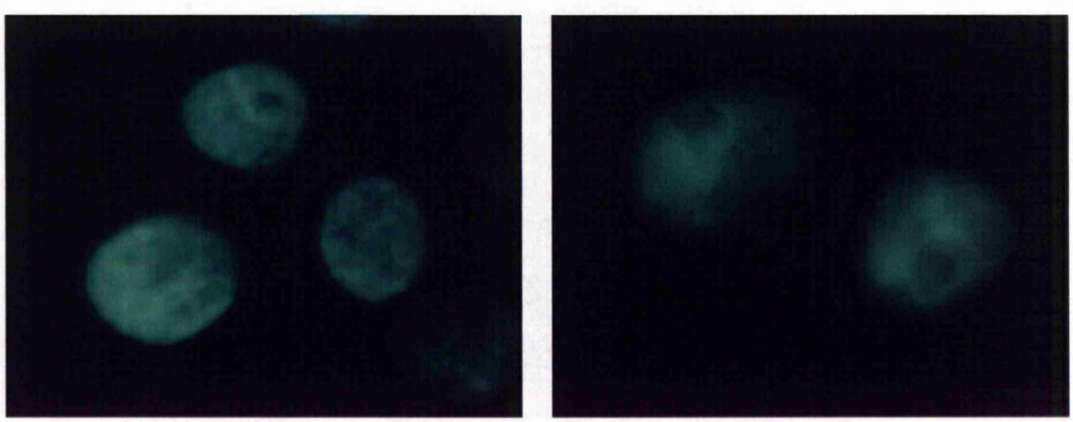
SWD20 - mycoplasma-free cells



SWK3 - mycoplasma free-cells



SW837 - mycoplasma free-cells



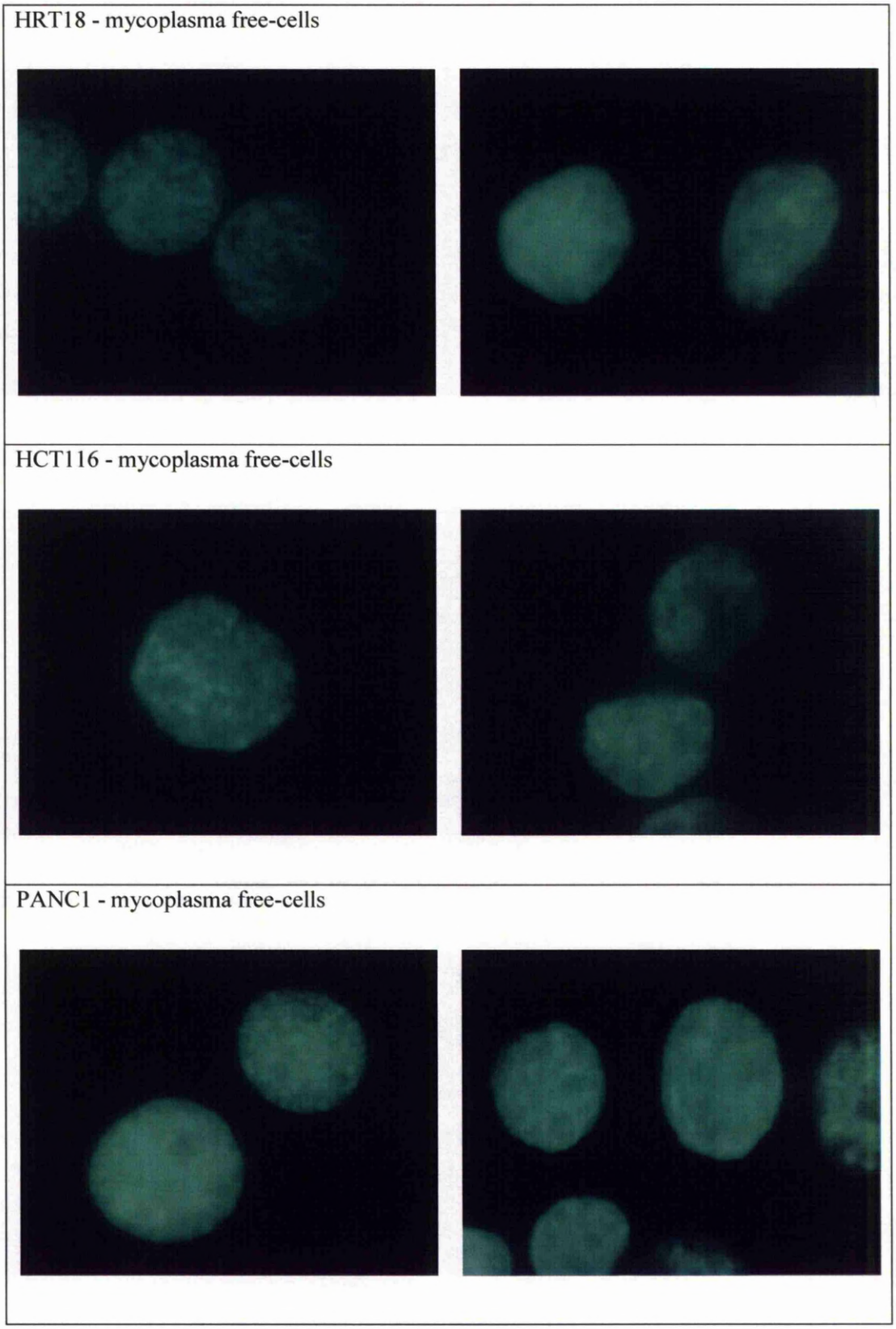


Figure 6: Mycoplasma screening on cell lines used in this study.

3.2.4 Western blotting

3.2.4.1 Cell lysate preparation

SDS cell lysis buffer	7 mL of distilled water, 2 mL of 10 % SDS solution, 1 mL of 1 M Tris pH 6.8, 1 tablet of protease inhibitor cocktail, ± 1 tablet of phosphatase inhibitor cocktail tablet (for probing phopho- proteins).

Box 3: Reagents used in cell lysis.

Cells were washed twice with PBS and centrifuged at 1300 rpm for 8 minutes to obtain a pellet. 100-300 µL of SDS cell lysis buffer (Box 3) was then added to the pellet, and then sonicated. Samples were then spun at centrifuge at 10,000 rcf at 4 ^{0}C for 10 minutes, with the consequent supernatant kept for western blotting and the pellet discarded.

3.2.4.2 Bicinchoninic acid (BCA) protein assay to measure cell lysate protein concentration

BCA™ Protein Assay kit was used to measure cell lysate protein concentration. This protein assay is a detergent-compatible formulation based on BCA for the colorimetric detection and quantification of total protein. The method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous action (Cu^{2+}) using a unique reagent containing BCA. This water soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (25-2,000 µg/mL).

To prepare diluted albumin standards, the content of one albumin standard ampoule at 2000 µg/mL (provided in the assay kit) was diluted serially using SDS cell lysis buffer to produce a set of diluted standards (Table 11). Working reagent was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of Reagent B. 50 µL of each sample usually diluted (1:5-1:100) and standards A-I were added to each cuvette, followed by 1 mL of working reagent. This mixture was then covered and incubated for 2 hours at room temperature. The standards were read on the spectrophotometer using the BCA function. Following standardisation, the samples were then read to determine the protein concentration of each sample.

Vial	Volume of diluents (µL)	Volume of albumin solution	Final albumin concentration (µg/mL)
A	0	300 µL of albumin ampoule	2000
B	125	375 µL of albumin ampoule	1500
C	325	325 µL of albumin ampoule	1000
D	175	175 µL of Vial B	750
E	325	325 µL of Vial C	500
F	325	325 µL of Vial E	250
G	325	325 µL of Vial F	125
H	400	100 µL of Vial G	25
I	400	0 µL	0

Table 11: Dilution of Albumin Standard to make up standards for BSA protein assay.

3.2.4.3 Tris-Glycine SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10 x Tris-Buffered Saline (TBS)	24.2 g Tris, 80 g NaCl, made up to 1 L and pH to 7.6 with hydrochloric acid.
Sample loading buffer	2 % of SDS, 10 % of glycerol, 60 mM of Tris-hydrocholoric acid (PH 6.8), 0.001 % of Bromophenol blue, 200 mM DL-Dithiothreitol.
5 % blocking milk solution	5 g of non-fat dry milk powder, made up to 100 mL with TBS Tween.
15 % Tris-glycine SDS-Polyacrylamide gel	To cast one gel (5 mL): 1.1 mL distilled water, 2.5 mL 30 % acrylamide mix, 1.3 mL 1.5 M Tris (pH 8.8), 0.05 mL 10 % SDS, 0.05 mL 10 % ammonium persulfate, 0.002 mL *N,N,N',N'*-Tetramethylethylenediamine.
12 % Tris-glycine SDS-Polyacrylamide gel	To cast one gel (5 mL): 1.6 mL distilled water, 2 mL 30 % acrylamide mix, 1.3 mL 1.5 M Tris (pH 8.8), 0.05 mL 10 % SDS, 0.05 mL 10 % ammonium persulfate, 0.002 mL *N,N,N',N'*-Tetramethylethylenediamine.
10 % Tris-glycine SDS-Polyacrylamide gel	To cast one gel (5 mL): 1.9 mL distilled water, 1.7 mL 30 % acrylamide mix, 1.3 mL 1.5 M Tris (pH 8.8), 0.05 mL 10 % SDS, 0.05 mL 10 % ammonium persulfate, 0.002 ml *N,N,N',N'*-Tetramethylethylenediamine.
5 % Stacking solution for Tris-glycine SDS-Polyacrylamide gel	To cast one gel (2 mL): 1.4 mL distilled water, 0.33 mL 30 % acrylamide mix, 0.25 mL 1.0 M Tris (pH 6.8), 0.01 mL 10 % SDS, 0.01 mL 10 % ammonium persulfate, 0.002 mL *N,N,N',N'*-Tetramethylethylenediamine.
10 x Running buffer	30 g Tris-base, 144 g Glycine, 10 g SDS, made up to 1 L with distilled water.
Transfer buffer	800 mL gel running buffer, 200 mL Methanol.
Membrane stripping buffer	20 mL 10 % SDS, 12.5 mL 1 M Tris-hydrochloric acid (pH 6.5), 0.7 mL β-mercaptoethanol, made up to 100 mL with distilled water.

Box 4: Reagents used to perform SDS-PAGE and Western Blotting.

The percentage of the acrylamide gel used depended on the size of protein of interest. Larger proteins can be resolved better on lower percentage gels. Glass plates (0.75 mm in thickness) were cleaned with 70 % ethanol, air dried and set up in a Bio-Rad kit. Resolving gel solutions (Box 4) were poured between the plates within 1.5cm of the top, covered with distilled water and left to polymerise. The distilled water was then removed with blotting paper and stacking gel solutions (Box 4) were added and a 10-well comb inserted. Once the stacking gel had polymerised, the comb was removed and the gel and plates were placed into a Mini Protean III electrophoresis chamber.

Generally 20 µg/well of cell lysates were calculated and prepared, and mixed with 1 x sample loading buffer (Box 4). The mixtures were then heated at 95 ^{0}C for 10 minutes for protein denaturation. Then, the samples were loaded onto the gel alongside a pre-stained molecular weight ladder. Gels were subjected to 55 mA for 60-90 minutes.

The running chamber was then dismantled, the stacking gel removed and the proteins in the resolving gel transferred onto a Hybond Enhanced Chemo-Luminescense (ECL) nitrocellulose membrane. The gel and membrane were sandwiched between 3mm Whatman chromatography paper and sponges. This assembled apparatus was then returned to the chamber which was filled with transfer buffer (Box 4), along with an ice block for cooling. The transfer took place on a magnetic stirrer which allowed circulation of transfer buffer, for 1 hour at 110 volts.

Following the transfer procedure, the membrane was removed and washed in Tris buffered saline tween (TBST) solution repeated twice each for 5 minutes.

The membrane was then blocked using 5 % blocking milk TBST solution for 90 minutes. Following blocking, the membrane was incubated overnight in 4 ^{0}C with primary antibody indicated in Table 12.

Following overnight incubation with primary antibody, the membrane was washed at room temperature with TBST for 4 cycles, 15 minutes each cycle. Then, the membrane was incubated with secondary horse radish peroxidase-linked antibody (dilution 1:2000, in 5 % milk TBST solution) for 90 minutes. Four cycles of TBST washes were repeated as before after incubation with secondary antibody. Using ECL Reagent Plus, equal quantities of the enhanced luminal and oxidising reagents were mixed and then applied to the membrane for 5 minutes. The membrane was then blotted dry, wrapped in cling film and secured in a Kodak light safe developing cassette. In the dark room, blots were exposed to medical x-ray film at a range of exposure lengths in order to optimise the imaging of various proteins being studied, and to gain an accurate appreciation of the expression levels. The film was consequently developed in Kodak developer and fixer for two minutes respectively. Finally the film was rinsed in tap water and allowed to air dry.

The membrane was then washed twice for 15 minutes with TBST and incubated with stripping buffer at 75 ^{0}C for 30 minutes. This was followed by two cycles of TBST washes before probing for β-actin or Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) overnight at 4 ^{0}C. Subsequent procedures were as described above. These steps were performed to check whether equal amount of protein were loaded into each lane (loading control) on the gel during the initial stage of the SDS-PAGE procedure.

Proteins	Gel	Primary antibody
HSP27	12 %	Mouse anti-HSP27 antibody, 1:5000 in 5 % milk TBST
Smad4	10 %	Mouse anti-Smad4 antibody, 1:500 in 5 % milk TBST
Phospho-Smad2	10 %	Rabbit anti-phospho-Smad2 antibody, 1:1000 in 5 % Bovine Serum Albumin (BSA) TBST
Phospho-Smad3	10 %	Rabbit anti-phospho-Smad3 antibody, 1:1000 in 5 % BSA TBST
Phospho-Smad1/5/8	10 %	Rabbit anti-phospho-Smad1/5/8 antibody, 1:1000 in 5 % BSA TBST
RAGE	12 %	1: 500 in 5 % milk TBST
β-actin (as loading control)	-	Mouse anti-β-actin antibody, 1:10000 in 5 % milk TBST
GAPDH (as loading control)	-	Rabbit anti-GAPDH antibody, 1:500 in 5 % milk TBST

Table 12: Gel electrophoresis for detection of proteins.

3.2.5 Transient HSP27 and Smad4 depletion/knockdown

HSP27 and Smad4 knockdowns were performed using siRNAs (all at 10 nM) with target sequences as below:

- Human HSP27-targetting-siRNA 1: GGCAGGACGAGCAUGGCUAUU
- Human HSP27-targetting-siRNA 2: CAAGUUUCCUCCUCCCUGU
- Human Smad4-targetting siRNA 1: GUGUGCAGUUGGAAUGUAA
- Human Smad4-targetting siRNA 2: GUACAGAGUUACUACUUAG
- Human non-targeting siRNA control 1: GGACGCAUCCUUCUUAAUU (kind gift of Dr Mark Boyd, Reader in Molecular Biology, University of Liverpool).
- Human non-targeting siRNA control 2: sequence not provided by Dharmacon company.

Cells were harvested, washed, counted, and plated onto 6-well plates. Routinely, $1x10^5$ cells were plated into each well to achieve cell density of ~60-70 %. Approximately 3 mL of culture media was added into each well and the plate incubated at 37 ^{0}C for 24 hours.

The following day, siRNAs and lipofectamine 2000 mixtures were prepared. Briefly, for transfection of each well, 4 μL of lipofectamine 2000 was added to 200 μL of Optimem I in one 15 mL tube, and siRNAs (10 nM for both HSP27 and Smad4 knockdown) were added to 200 μL of Optimem I in another 15 mL tube. These two tubes were incubated for 5 minutes in the hood before mixing their contents together and incubating for further 20 minutes.

The 6-well plates were retrieved from the incubator, the wells washed with PBS and 2.6 mL of antibiotic-free culture media was added into each well. The

siRNA and lipofectamine 2000 mixture was then added to the wells in a drop-wise manner. The plates were returned to the incubator for 48-96 hours and harvested for further experiments. Knockdowns were confirmed using western blotting.

3.2.6 Gamma irradiation to cells

Cells were harvested into either 50 mL polypropylene tubes or alternatively, 200,000 cells were plated into 10 cm^2 flat-sided tube flasks before exposure to a gamma source – Cs^{137} (Gamma Cell 1000, Atomic energy of Canada Ltd, at the Division of Immunology, University of Liverpool). At the current state of decay, 21 seconds exposure correlated to 1 Gray (Gy) of irradiation. Cells were then collected for further experiments.

3.2.7 Cell sensitivity and proliferation assay: The MTS assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), similar to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), is a yellow water-soluble tetrazolium dye that is reduced by live cells to an insoluble purple formazan product. The amount of MTS-formazan produced can be determined spectrophotometrically once solubilised in a suitable solvent. This provides an indirect measurement of the number of living cells.

Three chemotherapeutic agents were selected for experiments: 5-fluorouracil (5-FU), Oxaliplatin and Irinotecan. Oxaliplatin was diluted in PBS whereas 5-FU and irinotecan were diluted in DMSO as per the manufacturer's instruction.

Cells were harvested and plated into 96-well plates. Routinely, 3,000 cells in a volume of 100 μL were plated per well. The following day, variable concentrations of chemotherapy agents in a volume of 100 μL were added into the wells, to the final volume of 200 μL per well and incubated for 96 - 120 hours depending on the chemotherapeutic agents and cell lines. Following this, the MTS Assay Kit (EZ4U) was used according to the manufacturer's instruction. Briefly, 2.5 mL of activator was added to the substrate. Then, 20 μL of the mixture was added into each well. The plates were then immediately read at 450 nm with the biophotometer machine to determine the 0 hour readings. The plates were subsequently read every hour for 4-5 hours. Using the 450 nm measurements, graphs were then plotted to determine the IC_{50}.

To determine cell proliferation in general, MTS assays were performed as described above with the exception that cells were suspended in 200 μL of culture media into each well, and readings were performed at 24, 48 and 72 hours to measure cell proliferation over different periods of time. Recombinant S100A8-GST and S100A9-GST at concentrations of 0.4 μg/mL and 2 μg/mL were prepared in culture media containing 1 % FBS, the controls were culture media containing 1 % FBS, 10 % FBS, and GST.

For both cell sensitivity and cell proliferation assays, experiments were performed at least twice with five wells utilized for each treatment.

3.2.8 Flow cytometric cell cycle analysis

Cells ($0.5\text{-}1x10^6$) were harvested and washed twice in PBS (centrifuged 1000 rpm, 5 minutes each). PBS was removed, and the cell pellet re-suspended to

create a single cell suspension. 5 mL of ice cold 70 % ethanol (stored at -20 ^{0}C) was added drop-wise while gently vortexing. The samples were then incubated on ice for 60 minutes. Samples were then centrifuged at 1000 rpm for 5 minutes, washed twice in PBST to remove residual ethanol and re-suspended after each wash cycle. After pelleting the cells for the final time, PBST were removed and 100 µL of Ribonuclease A (10 mg/mL) was added and cells re-suspended. The samples were incubated on ice for 5 minutes, followed by the addition of staining solution containing 850 µL of PBS and 50 µL of propidium iodide (1 mg/mL) into each sample. The samples were incubated on ice in the dark for 30 minutes. The stained wells were then analysed using a FACScan flow cytometer (Beckman Coulter) (at the Division of pathology, University of Liverpool) and subsequently by WinMDI 2.9 software.

3.2.9 In-vitro cell motility/migration (chemotaxis) measurement – The modified Boyden Chamber assay

To set up a modified Boyden Chamber (Figure 7), a cell culture insert with PET membrane transwell 8µm pores was placed onto each well of a 24-well plate as an upper chamber. Cells (5000 for PANC1, 50,000 for SW480/SWK3/SWD20/HCT116, 100,000 for SW837/HRT18) were harvested and plated in 500 µL of 1 % FBS culture media in the upper chamber whereas the lower chamber contained 750 µL of cell culture media. Recombinant S100A8-GST and S100A9-GST at concentrations of 0.4 µg/mL and 2 µg/mL were prepared in culture media containing 1 % FBS, the controls were culture media containing 1 % FBS, 10 % FBS, and GST. These were plated in the lower chamber. For HSP27 experiments, 10 % FBS culture media were plated

in the lower chambers. The chambers were incubated at 37 ^{0}C for 18 hours at which time non-migrating cells were removed from the upper surface of inserts with a cotton swab. Cells which had migrated through the pores to the under-surface of the insert membrane were fixed and stained using Diff-Quick Stain Kit, the membrane was cut out and mounted onto glass slides. Using a microscope at 40 x magnification, stained cells were counted, n=2 inserts per treatment, and the average number of migrated cells calculated. Experiments were performed at least twice.

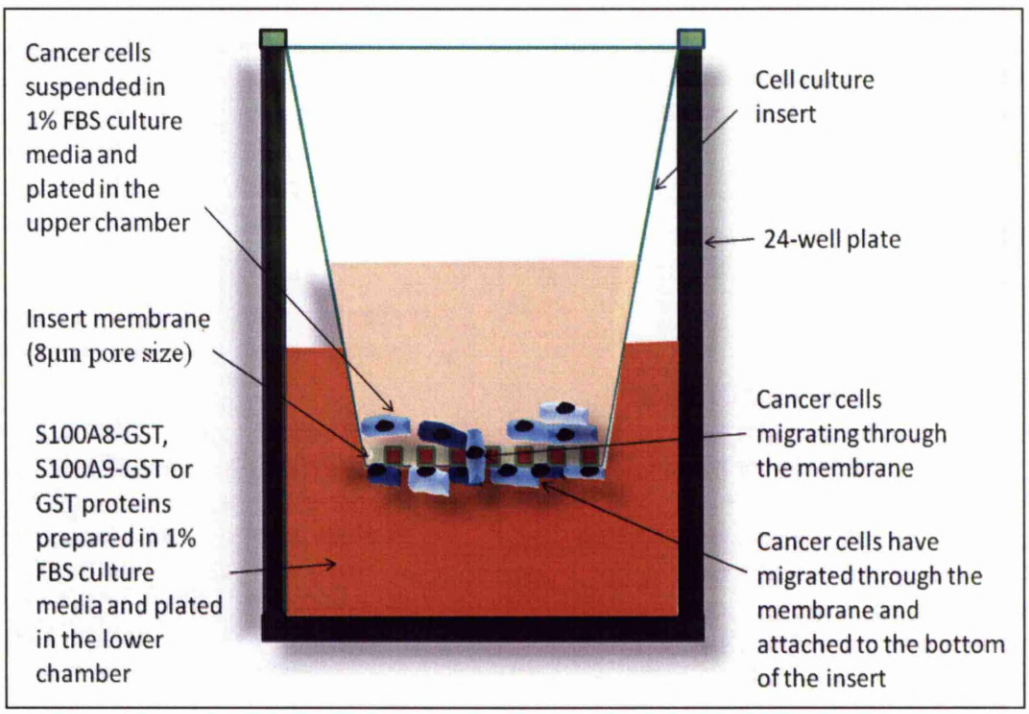


Figure 7: Modified Boyden Chamber.

3.2.10 Apoptosis assay - Annexin-V/Propidium Iodide staining and flow cytometry analysis

Cells (~$1x10^6$) were harvested and centrifuged at 1000 rpm for 5 minutes at 4 ^{0}C. The pellet was then washed twice with PBS and re-suspended in 1 mL of 1 x Annexin V binding buffer (Annexin V-FITC Apoptosis Assay Kit) and kept on ice. 500 µL of each sample was aliquoted into a flow cytometer tube, and 5 µL of FITC conjugated Annexin V and 10 µL of propidium iodide were added. This mixture was incubated on ice for 10 minutes in a dark box to prevent photo bleaching, after which the samples were ready to be analysed by FACSan. The data obtained were analysed by WinMDI 2.9 software.

For this experiment, staurosporine (1 µg/mL) was initially incubated with cells for 12 hours, to induce cell apoptosis as a positive control. This was used to set up the flow cytometric parameters and adjust for channel colour compensation.

3.2.11 The generation of GST-tagged S100A8 and S100A9 proteins

Luria-Bertani (LB) media	10 g Bacto-tryptone, 5 g yeast extract, 10 g sodium chloride, made up to 1 L with distilled water, pH to 7.0 with sodium hydroxide.
SLIP buffer	5 mL of 50 mM HEPES, 10 mL glycerol, 0.1 mL of Triton, 15 mL of 150 mM sodium chloride, made up to 100 mL with distilled water.
Elusion buffer	2.5 mL 1 M Tris pH 8.0, 0.154 g Glutathione (reduced), made up to 50 mL with distilled water.
Coomasie stain solution	2.5 g Coomasie blue G, 450 mL methanol, 100 mL acetic acid, made up to 1 L with distilled water.
Destaining buffer	300 mL methanol, 100 mL acetic acid, made up to 1 L with distilled water.

Box 5: Reagents used in the generation of recombinant proteins.

Plasmids encoding S100A8-GST and S100A9-GST fusion proteins[196] were the kind gift of Y. Maru, Tokyo Women's Medical University School of Medicine, Japan and were transformed into *Escherichia coli* by Mr Adnan Sheikh (an MD student, Division of Surgery and Oncology, University of Liverpool). For induction of protein expression, bacteria were cultured in Luria-Bertani media (Box 5, containing ampicillin 50 mg/mL) until they reached an optical density of 0.8 at 600 nm. Isopropyl-b-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and cultures incubated for a further 4 hours. The cultures were then centrifuged at 3,000 rpm for 8 minutes in 4 ^{0}C. GST fusion proteins were extracted by lysing the bacteria pellet in SLIP buffer (Box 5) using a sonicator, followed by incubating the supernatant with glutathione sepharose beads on an oscillator at 4 ^{0}C for 2 hours. The samples were then centrifuged, the supernatants were kept. 20 mL of ice cold PBS were added for washes. The samples were then aliquoted into the columns, with initial 1 mL of ice cold PBS added to the pellet, gently mixed. Proteins were then eluted and collected using 4 mL of elusion buffer (Box 5) into each column. The quantity of recombinant GST-S100A8 and GST-S100A9 was assessed by performing Coomasie-stained 1D SDS-PAGE against serial dilutions of a reference standard of BSA for comparison (1-15 μg/lane) followed by densitometric evaluation of the Coomassie-stained gels using TotalLab software.

The methodology for running Coomassie-stained gels for S100A8-GST and S100A9-GST proteins were similar to Tris-glycine gels as described in section 3.2.4.3. Following running of the Tris-glycine gels, Coomasie stain solution was added to the gels and incubated for at least one hour before de-staining buffer was added to visualise the presence of stained bands.

3.2.12 Cellular phospho-Smad expression and blocking of receptor of advanced glycation end products (RAGE)

Cells were cultured for 48 hours in serum free medium supplemented with bovine serum albumin (10 μg/mL) and human insulin (5 μg/mL) and then treated with S100A8-GST, S100A9-GST, GST or TGF-β (10 ng/mL) (as positive control) for 1 hour, followed by cell collection for western blotting as described above. For RAGE blocking, cells were treated with RAGE-blocking antibody (40 μg/mL or 80 μg/mL) for 1 hour before the addition of recombinant proteins.

3.2.13 Cellular immunofluorescence

Cells on glass slides were fixed with 4 % paraformaldehyde for 15 minutes at room temperature, washed with PBST, and then permeabilised with 0.1 % Triton X-100 for 10 minutes. Further washes with PBST were performed, followed by incubation with 10 % goat serum for 30 minutes. Rabbit anti-phospho-Smad2/3 antibody (1:50 dilution in 3 % BSA in PBST) was added and incubated overnight.

The following day, slides were washed three times with PBST (5 minutes each), followed by incubation with secondary goat fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:100 dilution in 3 % BSA in PBST) together with phalloidin (1: 500 dilution, for labelling actin filaments) for 60 minutes in the dark. Further washes with PBST were performed before slide covering with mowiol and a cover slip. Slides were analysed using a fluorescence microscope at the Division of Pathology, University of Liverpool.

3.2.14 Statistical analysis

Comparisons between two groups were made using non-parametric continuity corrected chi-square test or chi-squared test, when more than two groups were analyzed. Continuous variables were compared using the Mann-Whitney *U* or the Wilcoxon signed rank test. To evaluate patient survival, life tables were constructed and Kaplan Meier curves plotted. Cancer specific survival was measured from date of initial surgery to date of death, counting death from colorectal cancer as the end point. Multivariate Cox regression analysis was conducted using a stepwise forward selection approach starting with the most significant variable on univariate analysis and including every variable with a p value of <0.10.

A power calculation was performed based on the observed survival difference of 30% at 5-years between Dukes' C cancer patients treated with surgery followed by adjuvant chemotherapy and patients treated with surgery only[75, 293], and would have a power of 0.80 at a 5% level of statistical significance. This indicated that a minimum sample size of n = 44 in each arm (high or low HSP27 expression cancers) was required, assuming that cancer patients with high tumoural HSP27 treated with surgery and adjuvant therapy had similar survival compared with patients treated with surgery only.

To analyze data from laboratory experiments, continuous variables were compared using the student *t* test and were expressed as mean. All analyses were performed using Statview Version 5.01 (SAS Institute Inc). A *P* -value of less than 0.05 was considered significant.

CHAPTER 4:

THE ROLES OF S100A8 AND S100A9 AND THEIR RELATIONS TO TUMOURAL SMAD4 IN CARCINOGENESIS

4.1 Results

4.1.1 Generation of recombinant GST-tagged S100A8 and S100A9 protein.

S100A8-GST and S100A9-GST fusion proteins were generated and purified from *E. coli* as described in Chapter 3 section 3.2.11. The Coomasie-stained gels shown in Figure 8 confirmed the generation of S100A8-GST and S100A9-GST proteins. This was followed by a densitometric evaluation of Coomasie stained gels with known concentration of albumin standards, followed by graph plotting of albumin standards that allowed concentrations of S100A8-GST and S100A9-GST protein concentrations to be determined. At least 2 batches of each protein were generated and utilised in the subsequent experiments.

4.1.2 The effects of Smad4 status on S100A8 and S100A9-induced migration activity

S100A8 and S100A9 are secreted chemokines, which could affect tumour cells in their vicinity. Based on our recent observations that the microenvironment of Smad4-negative colorectal tumours had significantly reduced numbers of S100A8-expressing cells compared to the microenvironments of Smad4-positive tumours[136], we questioned whether the expression of Smad4 in cancer cells affected their response to exogenous S100A8 and S100A9.

The chemoattractive functions of these proteins on tumour cells were firstly examined *in vitro*. The addition of purified S100A8-GST and S100A9-GST fusion proteins at concentrations of either 0.4 μg/mL or 2 μg/mL significantly

increased the migration activity of the rectal cancer cell line SW837, the colon cancer cell line SW480, and the pancreatic cancer cell line Panc-1 ($P < 0.001$, Figure 9) compared to the addition of purified GST control protein. The addition of both S100A8-GST and S100A9-GST proteins simultaneously did not significantly increase the response over proteins added individually (Figure 9).

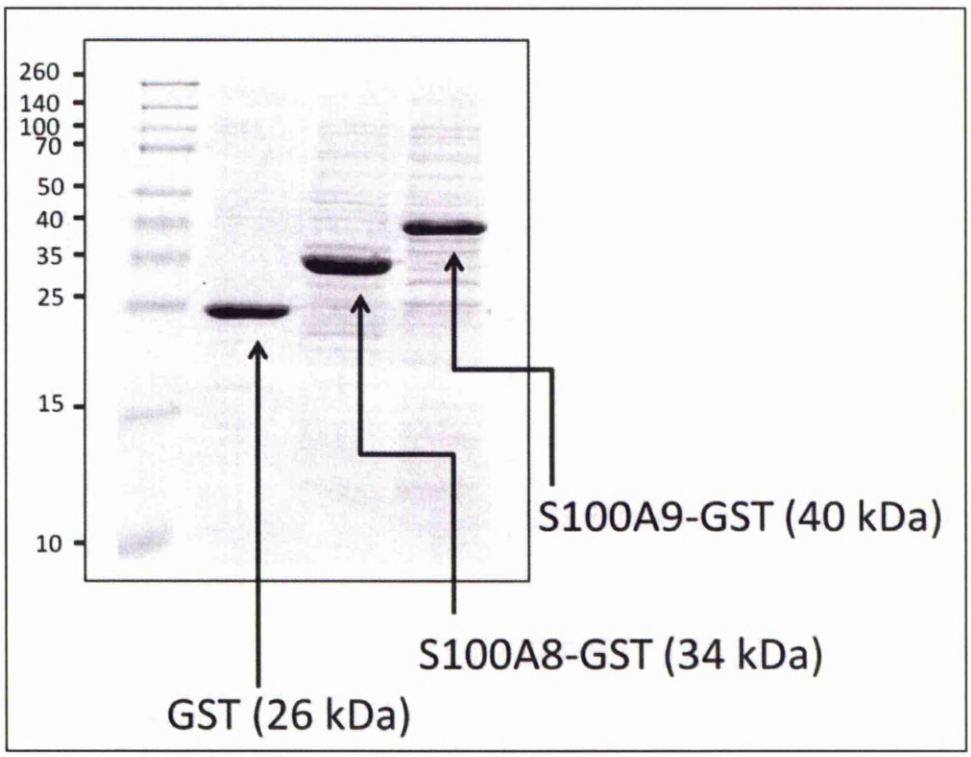


Figure 8: Colloidal Coomassie Blue stained polyacrylamide gel showing molecular weight markers and GST-tagged recombinant proteins, including GST, GST-S100A8 and GST-S100A9. The purity of the recombinant proteins was examined using densitometric evaluation of the Coomasie blue-stained immunoblot of the proteins against the background staining. This has shown the purity to be 85 % for S100A8-GST, 86 % for S100A9-GST, and 88 % for GST.

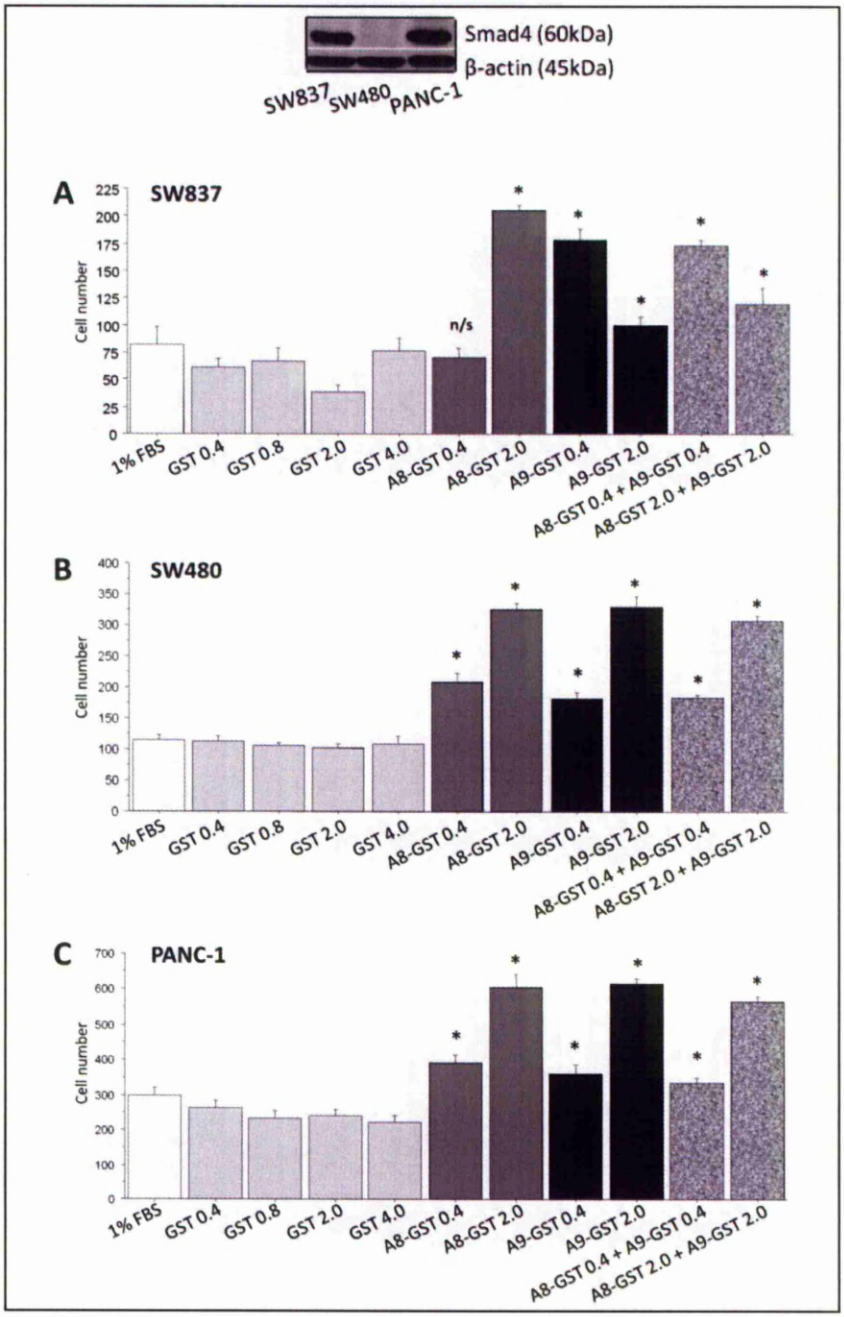


Figure 9: Migratory activities of cells in response to S100A8 and S100A9 proteins. Cell counts of indicated cell lines treated with S100A8-GST (A8-GST), S100A9-GST (A9-GST), S100A8-GST+S100A9-GST, or GST at concentrations of 0.4 or 2.0 µg/mL, in medium containing 1 % FBS. ** = $P<0.001$, * = $P<0.05$, n/s=non-significant. The Smad4 status of cells is shown as insets on Western blots. The results are representative of at least 2 independent experiments.

Both Panc-1 and SW837 cells express Smad4 (see inset Figure 9). To determine whether the Smad4 status of cells influenced their response to S100A8 or S100A9, Smad4 expression was depleted in these cell lines and the cells' migration activity in response to S100A8 and S100A9 was then examined. An approximate 50 % decrease in basal migration in Panc-1 cells compared to control siRNA treated cells was observed (Figure 10A). Smad4 knockdown totally suppressed migration responses towards S100A8. In contrast, S100A9-treated cells retained an approximate two-fold increase in migration after Smad4 knockdown. Smad4 knockdown caused an almost total loss of basal and chemokine-induced migration of the less motile SW837 cells (Figure 10B).

SW480 cells do not express Smad4 (see inset Figure 9). Neomycin resistant clonal derivatives of SW480 cells, stably re-expressing Smad4 (SWD20) and negative control transfectants (SWK3) were used to determine whether restoration of Smad4 would alter responsiveness to S100A8 or S100A9. Both the Smad4-negative and Smad4-positive subclones had enhanced migration following incubation with S100A8-GST or S100A9-GST (Figure 11A). However, transient siRNA-mediated depletion of Smad4 from SWD20 cells resulted in a reduction in motility and a loss of responsiveness to S100A8-GST, but not S100A9-GST (Figure 11B). This was similar to the result obtained with Panc-1 cells (Figure 10A).

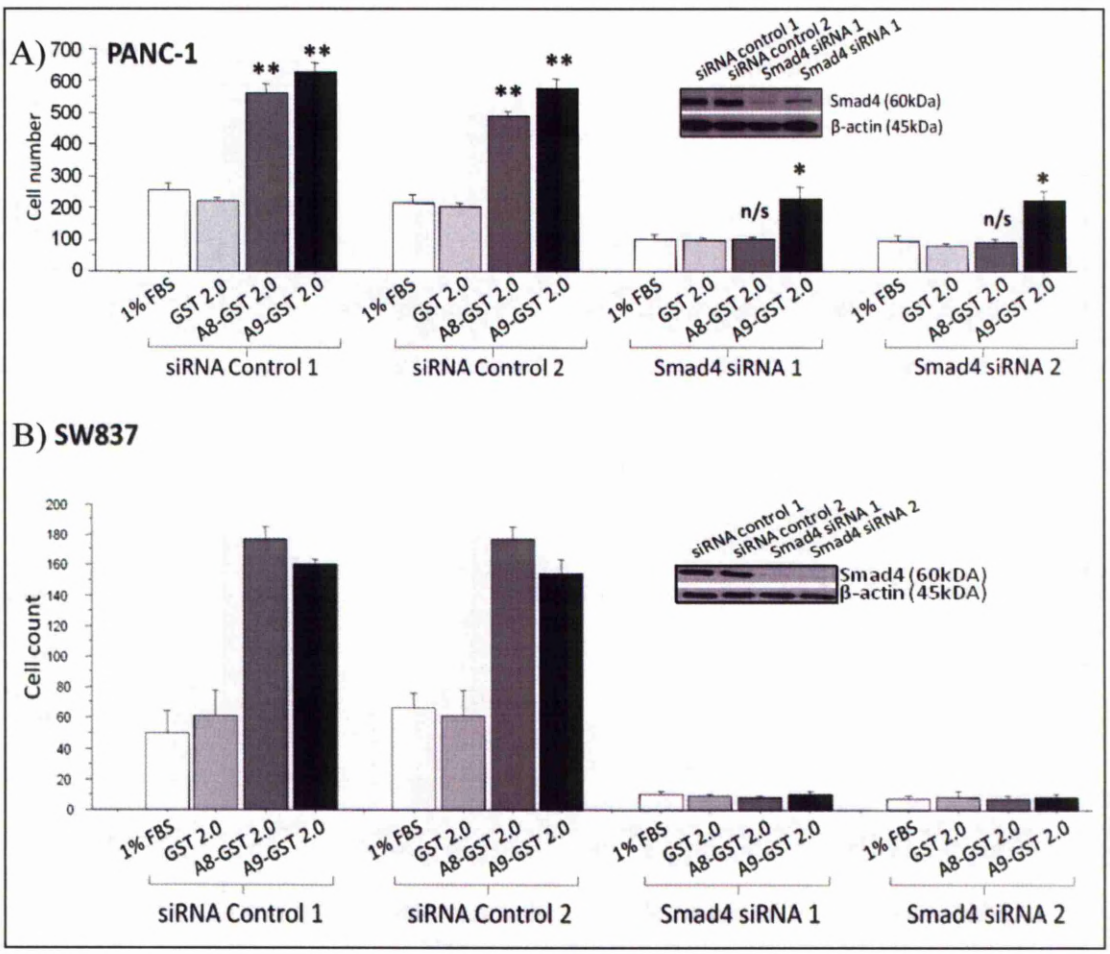


Figure 10: Migratory activities of PANC1 and SW837 cells in response to S100A8 and S100A9 proteins following Smad4 depletion. The cell counts of Smad4 depleted PANC1 cells (A) and SW837 cells (B), in response to exogenous S100A8 and S100A9. ** = $P < 0.001$, * = $P < 0.05$, n/s = non-significant. The Smad4 status of cells is shown as insets on Western blots. The results are representative of at least 2 independent experiments.

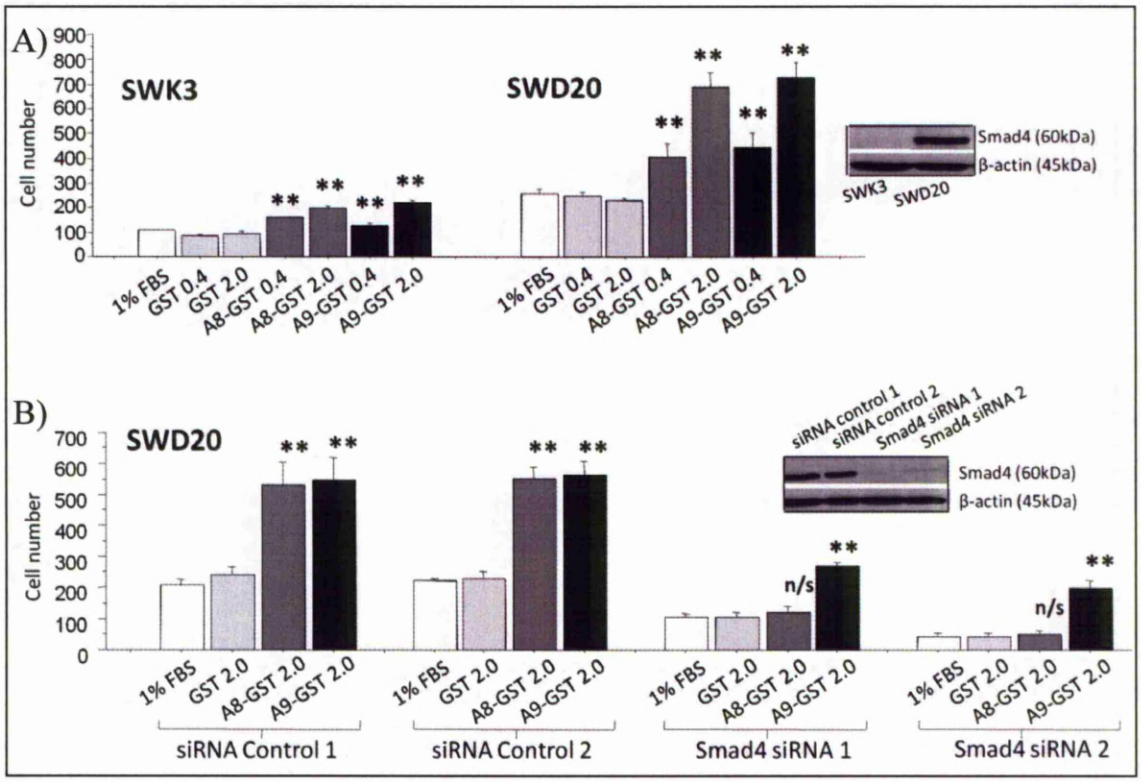


Figure 11: Migratory activities of clonal derivative of SW480 cells or Smad4 depleted cells in response to S100A8 and S100A9 proteins. Cell counts of clonal derivative of SW480 cells that stably re-expresses Smad4 (SWD20) and its Smad4-negative control (SWK3) (A), or Smad4 depleted SWD20 (B), in response to exogenous S100A8 and S100A9. ** = $P < 0.001$, * = $P < 0.05$, n/s = non-significant. The Smad4 status of cells is shown as insets on Western blots. The results are representative of at least 2 independent experiments.

4.1.3 The effects of Smad4 status on S100A8 and S100A9-induced proliferation.

Similarly, the effects of S100A8 and S100A9 on tumour cell proliferation were examined and the roles of Smad4 status in this context were explored. Incubation of SW837 and SW480 cells with 2 μg/mL of S100A8-GST or S100A9-GST resulted in modest, though statistically significant increases in MTS readings (Figure 12A & B). No increase was observed using 0.4 μg/mL recombinant proteins and Panc1 cells did not show an increased MTS response at all (Figure 12C). In the case of SW837 cells, the simultaneous addition of S100A8-GST and S100A9-GST proteins at 2 μg/mL significantly improved the response over proteins added individually (Figure 12A), although this effect was not observed with SW480 or Panc-1 cells (Figure 12B & C).

The Smad4-negative SW480 subclone, SWK3 showed enhanced proliferation in response to 2 μg/mL S100A9-GST (Figure 13A), whilst the Smad4 re-expressing subclone, SWD20, responded to both S100A8-GST and S100A9-GST, although the response to S100A9-GST was greater (Figure 13B). Following depletion of Smad4 from SWD20 cells, responsiveness to S100A8-GST was lost (Figure 13C). S100A9-GST continued to induce proliferation, although the extent of proliferation reached was not as great as that observed in control siRNA treated cells (Figure 13C). Depletion of Smad4 from Panc-1 cells gave a similar result (Figure 13D) although the increase in MTS readings in response to S100A9 was very small.

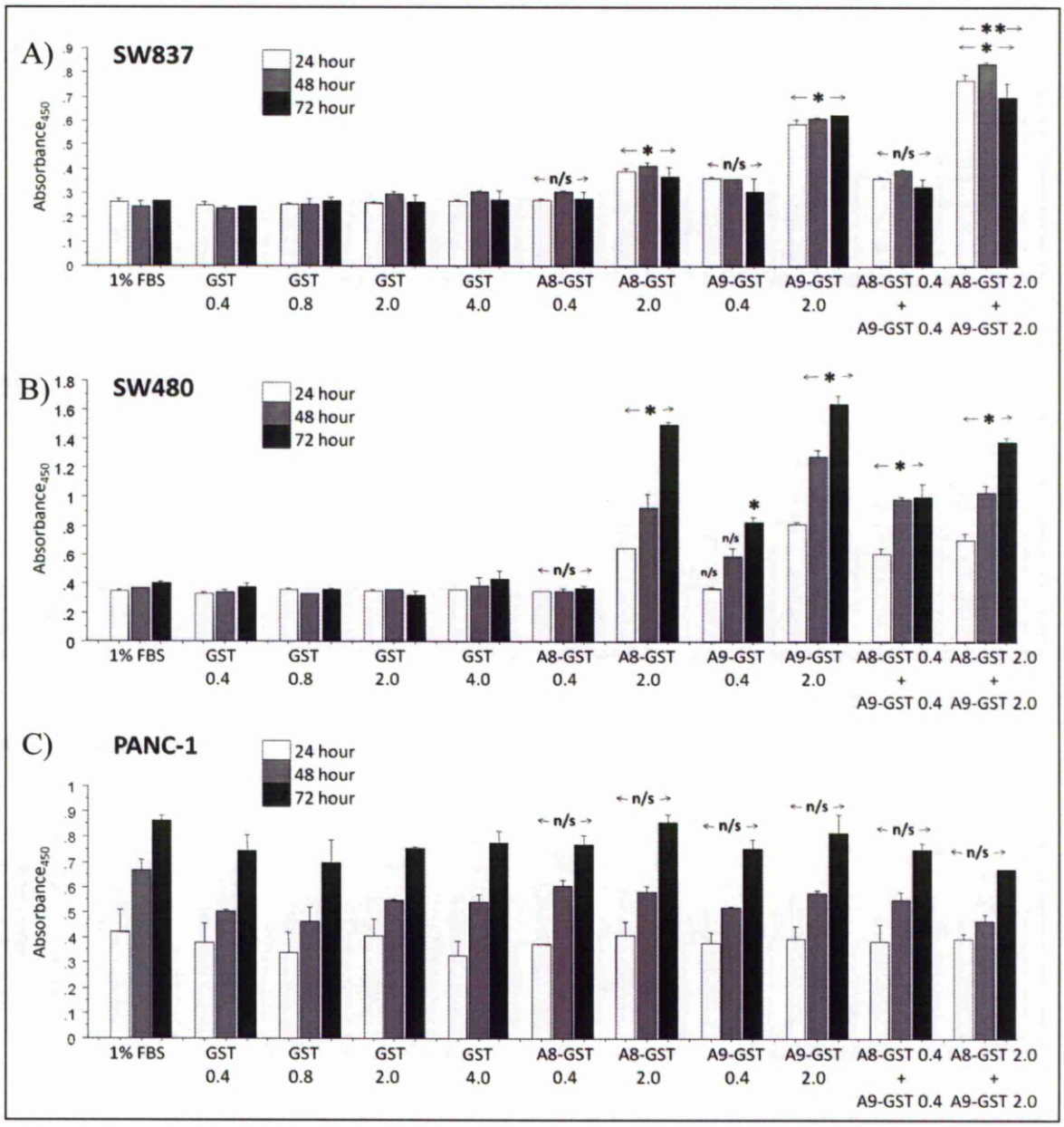


Figure 12: Proliferation of cells in response to S100A8 and S100A9. MTS readings of indicated cell lines treated with S100A8-GST (A8-GST), S100A9-GST (A9-GST), S100A8-GST+S100A9-GST, or GST at concentrations of 0.4 or 2.0 μg/mL, in medium containing 1% FBS. * = $P < 0.05$, n/s = non-significant, ** = the simultaneous addition of S100A8-GST and S100A9-GST proteins at 2 μg/mL significantly improved the response over proteins added individually. The results are representative of at least 2 independent experiments.

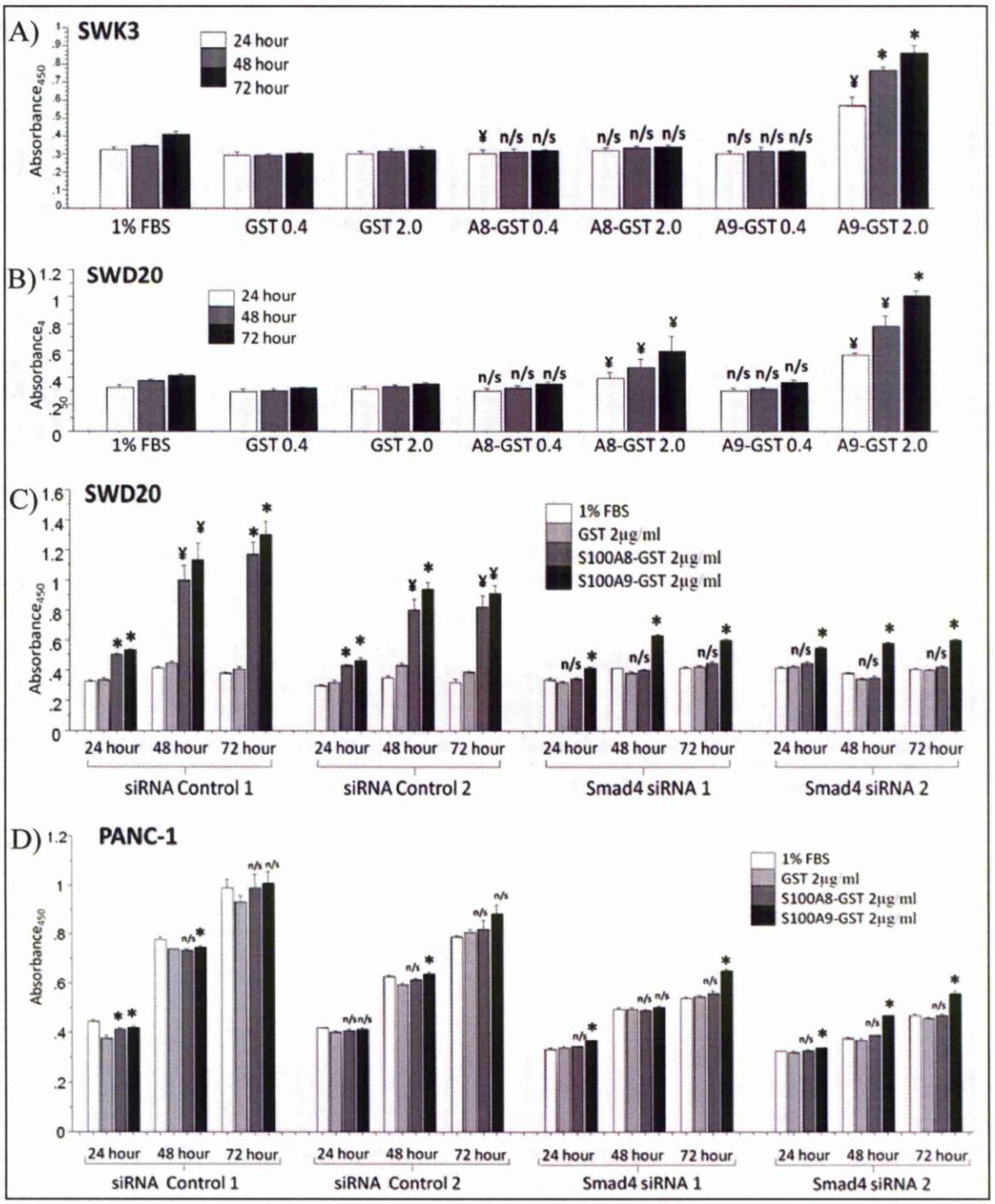


Figure 13: The roles of Smad4 status on S100A8 and S100A9-induced proliferation.MTS readings of A) SWK3 cells, B) SWD20 cells, Smad4 depleted C) SWD20 cells and D) PANC1 cells, in response to S100A8-GST (A8-GST), S100A9-GST (A9-GST), or GST at a concentration of 2.0 μg/mL. * = P<0.01, ¥ = $P < 0.05$. The results are representative of at least 2 independent experiments.

4.1.4 The effects of exogenous S100A8 and S100A9 on Smad4 signalling and RAGE

The results above indicated that the effects of exogenous S100A8 and S100A9 were influenced to an extent by the presence of Smad4 in tumour cells. To examine whether these proteins could signal via Smad4, cells were incubated for 1 hour with 2 μg/mL of S100A8-GST or S100A9-GST and the activation of members of the Smad pathway examined. Both recombinant S100 proteins alone, but not GST, induced increased levels of phospho-Smad2, phospho-Smad3, but not phospho-Smad1/5/8 in Panc-1 cells (Figure 14A). Furthermore, phospho-Smad2/3 was shown to accumulate in the nucleus following incubation with S100A8-GST or S100A9-GST, but not GST (Figure 14B).

S100A8/A9 heterodimers are known to stimulate cells through binding to the cellular receptor RAGE, although it is unclear whether the individual proteins are ligands for this receptor. RAGE expression was observed in the cell lines used in this study (Figure 15A). To determine whether S100A8 and S100A9 were activating the Smad pathway through RAGE, RAGE-blocking antibody was used at two concentrations, 40 μg/mL and 80 μg/mL. When Panc-1 cells were pre-treated with the RAGE-blocking antibody, a dose-dependent reduction of phopho-Smad2 and phospho-Smad3 levels was observed in response to S100A8-GST or S100A9-GST stimulation (Figure 15B).

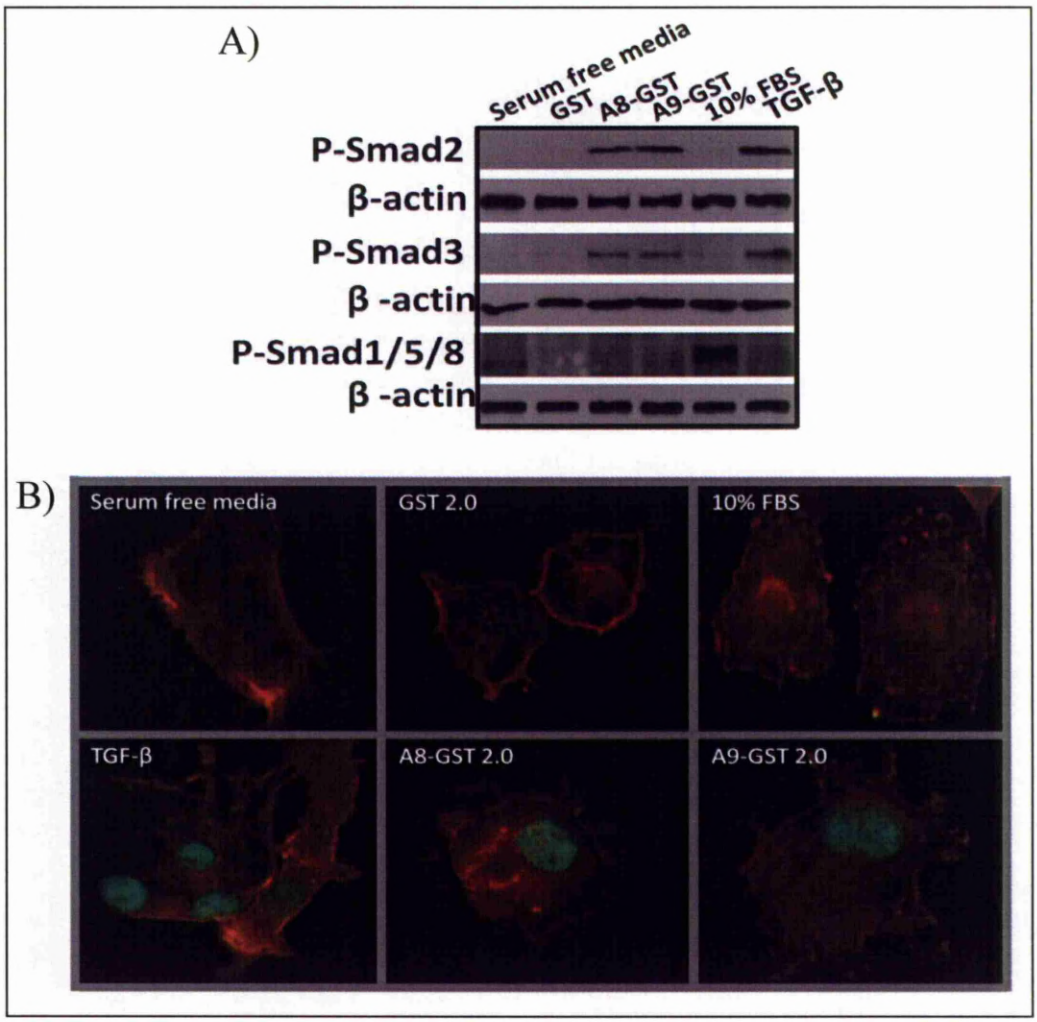


Figure 14: The effects of exogenous S100A8 and S100A9 on Smad4 signalling. A) Western detection of phospho-Smad2, phospho-Smad3, phospho-Smad1/5/8 levels following treatment of Panc-1 cells with 2 µg/ml of S100A8-GST (A8-GST), S100A9-GST (A9-GST) or GST. B) Cyto-immunofluorescence showing accumulation of phospho-Smad2/3-heteromeric complex to the nucleus (green) following 1 h treatment of PANC1 cells with 2 µg/mL of S100A8-GST or S100A9-GST. Actin filaments have been labelled with phalloidin (Red). TGF-β (10 ng/mL) and 10 % FBS were the positive control for phospho-Smad2/3 and phospho-Smad1/5/8 activation respectively.

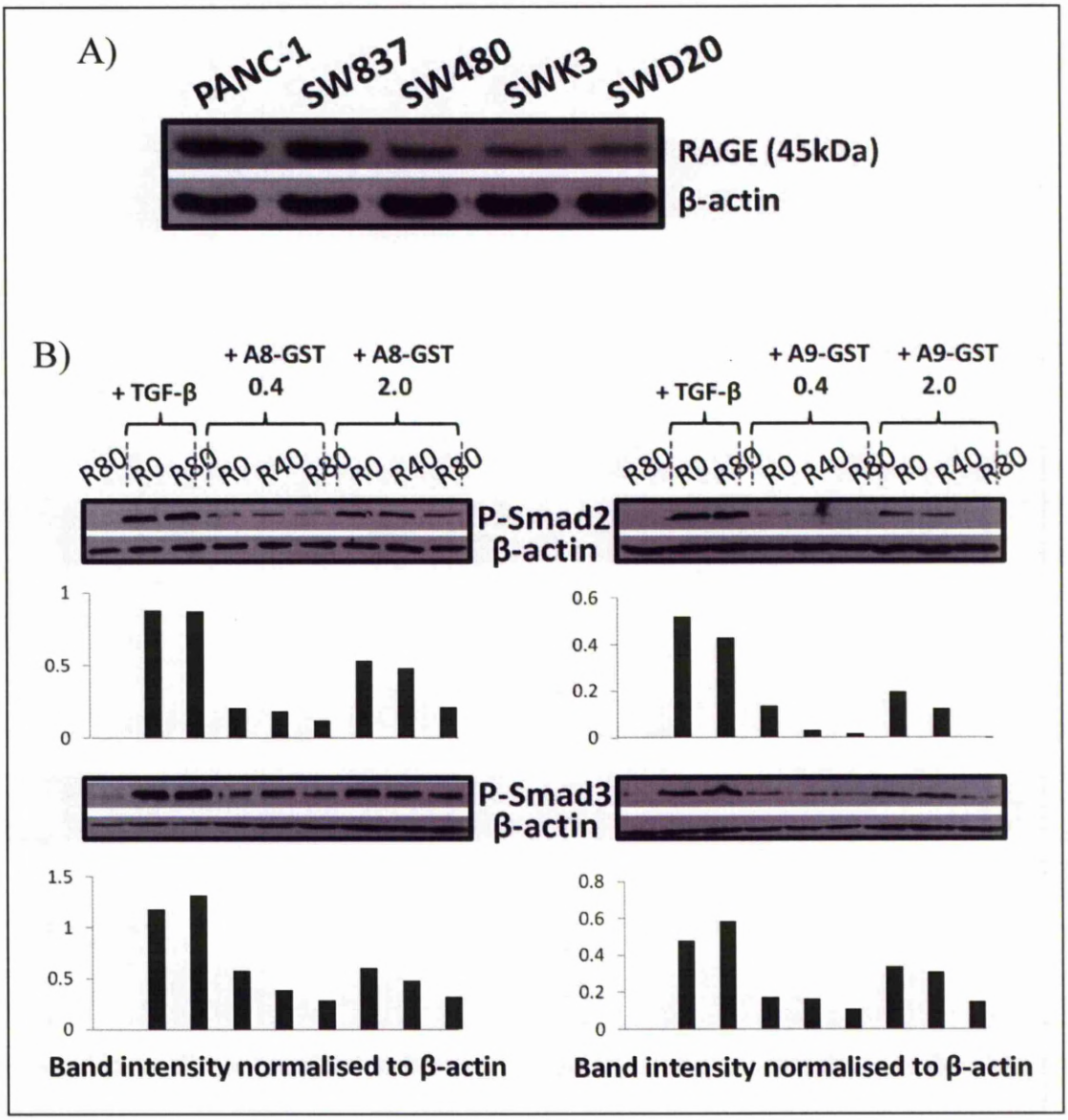


Figure 15: S100A8 and S100A9 activated the Smad pathway through RAGE. A) Western detection of RAGE expression in indicated cell lines. B) Western detection and densitometric representation of the levels of phospho-Smad2 and phospho-Smad3 which were reduced in Panc-1 cells pre-treated with RAGE-blocking antibody (R) (40 μg/mL and 80 μg/mL) before the addition of 0.4 μg/mL or 2 μg/mL of S100A8-GST or S100A9-GST.

4.2 Discussion

The inflammatory chemoattractants, S100A8 and S100A9, apart from serving as markers of phagocytes (intracellular S100A8/A9) or as biomarkers of inflammatory disease conditions (secreted extracellular S100A8/A9), are now recognized to play important roles themselves in the pathogenesis of inflammatory disorders and more recently in cancer[153].

Hiratsuka and colleagues[196] showed that S100A8/A9 were powerful chemoattractants, whose tumour-induced presence in the lungs of tumour bearing mice, could stimulate the migration of Lewis Lung carcinoma cells and B16 melanoma cells to that organ, where they formed secondary tumours. Coupled with the evidence that loss of Smad4 affects tumour microenvironment and promote invasion[226, 227], and our previous observation that the microenvironments of colorectal and pancreatic[137] tumours which lacked Smad4 had significantly reduced S100A8-expressing but not S100A9-expressing inflammatory cells, we sought to determine whether the response of tumour cells to S100A8/A9 was potentially influenced by their Smad4 status. Both S100A8 and S100A9 were highly chemotactic for rectal, colon and pancreatic cancer cell lines, regardless of whether the cells expressed Smad4 or not. In Smad4 expressing cells, such as Panc-1 cells or the SWD20 cells, where Smad4 expression has been stably restored, transient depletion of Smad4 expression was accompanied by a loss of responsiveness to S100A8-induced migration activity but not that of S100A9, suggesting that S100A8 elicits its response through a Smad4 dependent pathway. Transient Smad4 depletion, such as we undertook in our siRNA experiments, may not allow sufficient time for cells to adapt and to

allow S100A8 to signal in a Smad4-independent manner. Our observation of increased levels of phosphor-Smad2 and phospho-Smad3 in response to cell treatment with S100A8 and S100A9 provide supporting evidence that these proteins can activate the Smad4 signalling pathway.

S100A8/A9 induced proliferation of the cancer cell lines studied was also observed, although at concentrations higher that those required to induce migration activity. S100A8/A9 may contribute to apoptosis[153], however the apoptotic role of these proteins was not investigated in this study. Ghavami and colleagues[192] reported that S100A8 and S100A9 proteins promoted growth of human breast cancer and neuroblastoma cells and confirmed that these proteins activate the multi-ligand receptor, RAGE, triggering the MAP kinase signalling pathway. The mitogenic effects of S100A8/A9, coupled with previous observation in our research unit that high levels of A8/A9 infiltrate were associated with larger tumours suggest that the proteins may contribute to the growth of these tumours. Interestingly, while the Smad4-restored SWD20 cells showed increased proliferation in response to S100A8-GST and S100A9-GST, their Smad4-negative clonal counterpart, SWK3 cells showed increased proliferation in response to S100A9-GST only, suggesting that Smad4 is important in regulating the proliferative response to exogenous S100A8. Transient Smad4 knockdown in SWD20 and Panc-1 was accompanied by a loss of response to S100A8-GST. Thus, although the measured proliferation effects of S100A8/A9 were not as marked as their chemotactic effects, the dependence on Smad4 for S100A8 signalling was a recurrent theme. Our RAGE blocking experiments provide evidence that S100A8 and S100A9 activation of the Smad4 pathway occurs at least in part through RAGE. The ability of advanced glycation

end products to activate TGF-β signalling via RAGE and MAPKs has been established by Li and colleagues[294].

Interestingly, our data provides important evidence that S100A8 and S100A9 share similarity to TGF-β, in that they activate the Smad pathway and their effects can be Smad-dependent or Smad-independent, as revealed by our motility and proliferation assays. In normal epithelial and haematopoietic cells, TGF-β acts as a tumour suppressor with anti-proliferative property. In cancers, TGF-β can function as an oncogenic factor facilitating proliferation, angiogenesis, cell migration, invasion and metastasis. Several complex molecular mechanisms have been identified to allow cancer cells to evade from the tumour suppressive effect of TGF-β while switching to its oncogenic factor[295]. This double-edged sword effect of TGF-β is in a way, similar to S100A8/A9, where these S100 proteins are powerful apoptotic agents produced by immune cells but on the other hand, they have also been shown to facilitate tumour development, cancer invasion or metastasis. The molecular pathways associated with the dichotomous role of these S100 proteins have not been fully understood currently, while the signal transduction pathways of TGF-β (Smad-dependent and Smad-independent) have been increasingly elucidated.

A limitation of this study has been the use of recombinant S100A8 and S100A9 proteins produced in *E. coli*. Although we do not know whether these proteins existed as homo- or heterodimers in our experiments, purified recombinant S100A8 and S100A9 proteins can exist as homodimers, or they can form heterodimers, tetramers or oligomers depending on the presence of calcium[156]. Furthermore, the addition of both S100A8-GST and S100A9-GST proteins

simultaneously did not significantly increase the motility response over proteins added individually, although in the case of SW837 cells, the simultaneous addition of S100A8-GST and S100A9-GST proteins at 2 μg/mL significantly improved the proliferative response over proteins added individually, although this effect was not observed with SW480 or Panc-1 cells. It is not entirely clear to us why the addition of both proteins did not always enhance the effects observed. We can suggest two possible reasons: the individual proteins at the concentrations used have generated a maximum response for that concentration, such that the combined proteins cannot exceed that affect; the proteins when added in combination do not heterodimerise adequately due to interference by the GST portion. GST is a relatively large size with 220 amino acids (26kDa) and is fused to the N-terminus of a protein. GST-tagged recombinant proteins have been used extensively in research, with the advantage of maintaining functional integrity compared to the native protein while providing the sufficient quantities to further study structure and function. One recent study by Viterbo and colleagues[296] demonstrated that GST tagged recombinant protein from *E. coli* (pancreatitis associated protein) has similar functional immunomodulatory integrity as with the native protein. In all of our experiments GST protein alone was used as a control for the GST-tagged S100 proteins. We have shown that GST does not have an effect on cancer cell motility, proliferation or cellular Smad4 signalling, although some loss/interference in function due to the GST tag cannot be ruled out. Other researchers[192] (using untagged S100A8/A9 purified from human blood neutrophils) have also demonstrated some of our positive findings regarding the ability of S100A8 and S100A9 to promote motility and proliferation.

A second limitation of our study is that the physiological concentrations of S100A8 and S100A9 in the microenvironment of colorectal tumours are unknown. The concentrations of S100A8-GST and S100A9-GST used in our *in vitro* experiments (0.4 μg/mL and 2.0 μg/mL) are however in agreement with other research groups. For instance, Ghavami and colleagues[192] showed that S100A8/A9 has cell growth-promoting properties at concentration between 0 and 25 μg/mL. Consistent with our data, Turovskaya and colleagues[193] also demonstrated growth-promoting effect of S100A8/A9 at 1 ug/mL in colon cancer cells. Hiratsuka and co-workers[196, 197] used lower concentrations (pg/mL) to demonstrate motility-promoting effect.

Finally, the generation and use of recombinant proteins from *E. coli* raises the concern of impurity, in that soluble factors such as lipo-polysaccharide from the bacteria may produce, or contribute to the observed effects in our *in vitro* experiments. The research group in our unit is currently performing *in vitro* experiments using S100A8 and S100A9 antibodies along with the recombinant GST-tagged S100 proteins, further examining the functional aspects of these proteins. These experiments have shown that recombinant S100A8 and S100A9 proteins can be inhibited through the use of S100A8 and S100A9 antibodies respectively, suggesting the reliability of the observed functions of these recombinant S100 proteins.

In summary, we provide evidence on the cancer-promoting activity of S100A8 and S100A8 proteins, suggesting the roles of inflammatory cytokines in promoting tumour development/spread. Moreover, we have shown for the first time, that the effects of these proteins on cancer cells are in part related to the

Smad4 status of these cancer cells in orchestrating the crosstalk between cancer cell and its microenvironment.

CHAPTER 5:

THE CLINICAL RELEVANCE OF TUMOURAL HSP27 EXPRESSION AS A PREDICTIVE MARKER OF RESPONSE TO ADJUVANT THERAPY IN COLORECTAL CANCER

5.1 Result

5.1.1 Patient selection for analyses

To examine whether the level of tumoural HSP27 expression affects patient response to chemotherapy, neoadjuvant and adjuvant data for 404 colorectal cancer patients, for which the tumoural HSP27 levels had already been characterised (by Ms Elizabeth Tweedle) were obtained from the Clatterbridge Centre for Oncology, Liverpool, while the colorectal cancer-specific death data were obtained from the North West Cancer Intelligence Service, Liverpool.

Of these 404 patients, only the patients with Dukes' C cancers (n = 178), who would be routinely considered for adjuvant therapy were explored (Figure 16). While there were 50 patients that received either neoadjuvant, adjuvant or both, these patients were excluded from analyses as the treatments that they have received were not directly comparable. However, there were 58 patients who received only adjuvant 5-FU following surgery, while 70 patients did not receive any form of neoadjuvant or adjuvant therapy. Therefore, these two cohorts of patients were selected for further analyses.

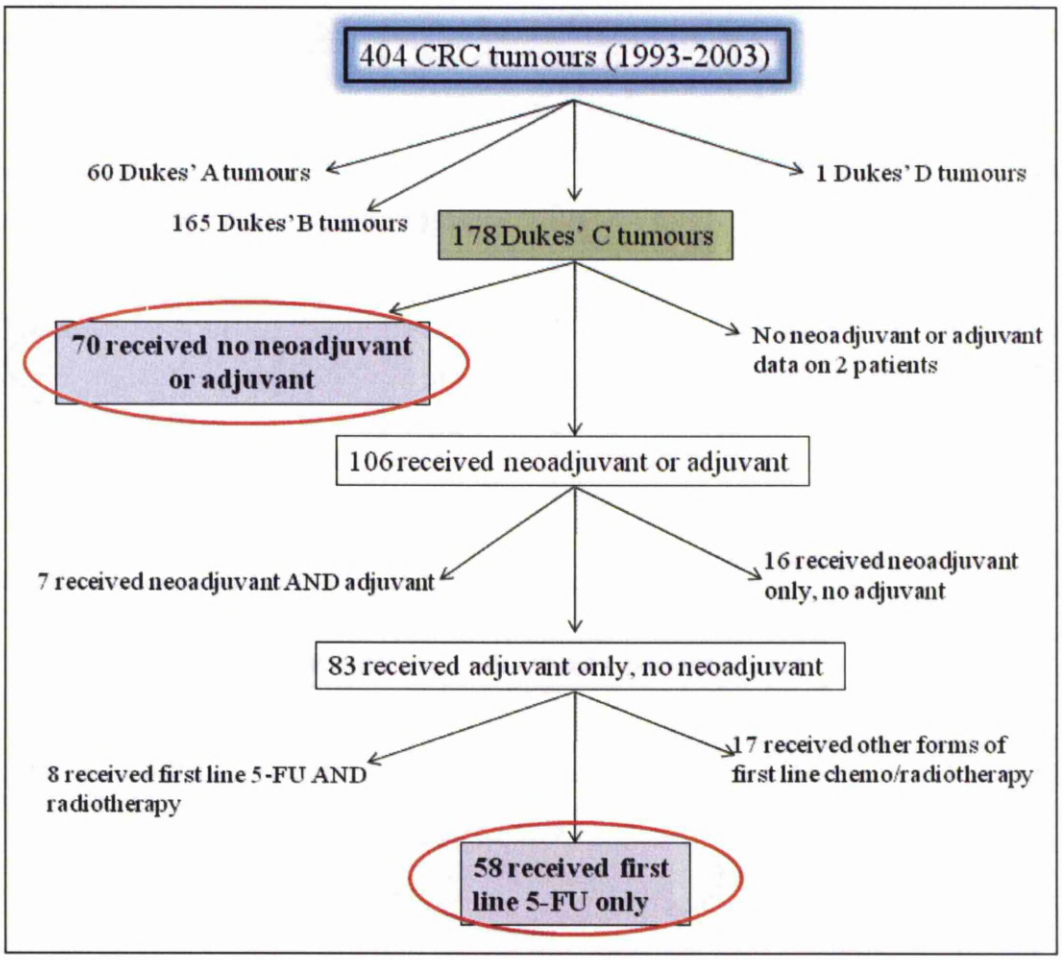


Figure 16: Patient selection for statistical analyses. Seventy patients treated with surgery and 58 patients treated with surgery followed by adjuvant 5-FU were selected for analyses.

5.1.2 Patient demographics and clinico-pathological features

The two cohorts of patients selected for analyses showed no significant difference in respect of the parameters of gender, tumour site, tumour size, degree of cancer cell differentiation, tumour resection margin, T-stage, N-stage or 5-year cancer-specific death rates (Table 13). However, there was a significant difference between the ages of the two patient groups. The median age of patient

in the study was 68 years. In the cohort of patients who underwent surgery only, a large proportion (74.3 %, 52/70) were 68 years or older, compared to patients treated with adjuvant 5-FU, where only 13/45 patients (28.9 %) were 68 years or older ($P < 0.0001$, Table 13).

	Dukes' C CRC, treated with Surgery only Total, n = 70	Dukes' C CRC, treated with Surgery + adjuvant 5-FU Total, n = 58	*P* value
Gender			
Male	33	38	0.232‡
Female	37	20	
Age (median = 68 [35-90])			
≥ 68	52	13	***< 0.0001‡***
< 68	18	45	
Tumour site			
Colon	42	29	0.257‡
Rectum	28	29	
Tumour Size (median = 50mm)			
≥ 50	39	32	0.951‡
< 50	31	26	
Degree of differentiation			
Well	1	0	0.289*
Moderate	61	55	
Poor	8	3	
Resection margin			
Positive	12	6	0.270*
Negative	58	52	
T-Stage			
I	1	2	0.171‡
II	4	7	
III	49	43	
IV	16	6	
N-Stage			
I	36	32	0.672‡
II	34	26	
5-year cancer-specific death			
Yes	33	28	0.898‡
No	37	30	

‡ Chi-squared test
* Fisher-exact test

Table 13: Patient demographics and clinico-pathological features.

Associations were sought between HSP27 levels and clinico-pathological parameters of gender, age, tumour site, tumour size, degree of cancer cell differentiation, resection margin, T-stage and N-stage (Table 14), although none were found. However, among patients treated with surgery and adjuvant 5-FU, there was a positive association between high tumoural HSP27 and death within 5 years. In this treatment group, 68.2 % (15/22) of patients had died within 5 years compared to only 36.1 % (13/36) patients with low tumoural HSP27 (P = 0.017, Table 14). For patients who underwent surgery only, there was no association between HSP27 levels and 5-year cancer specific death (Table 14).

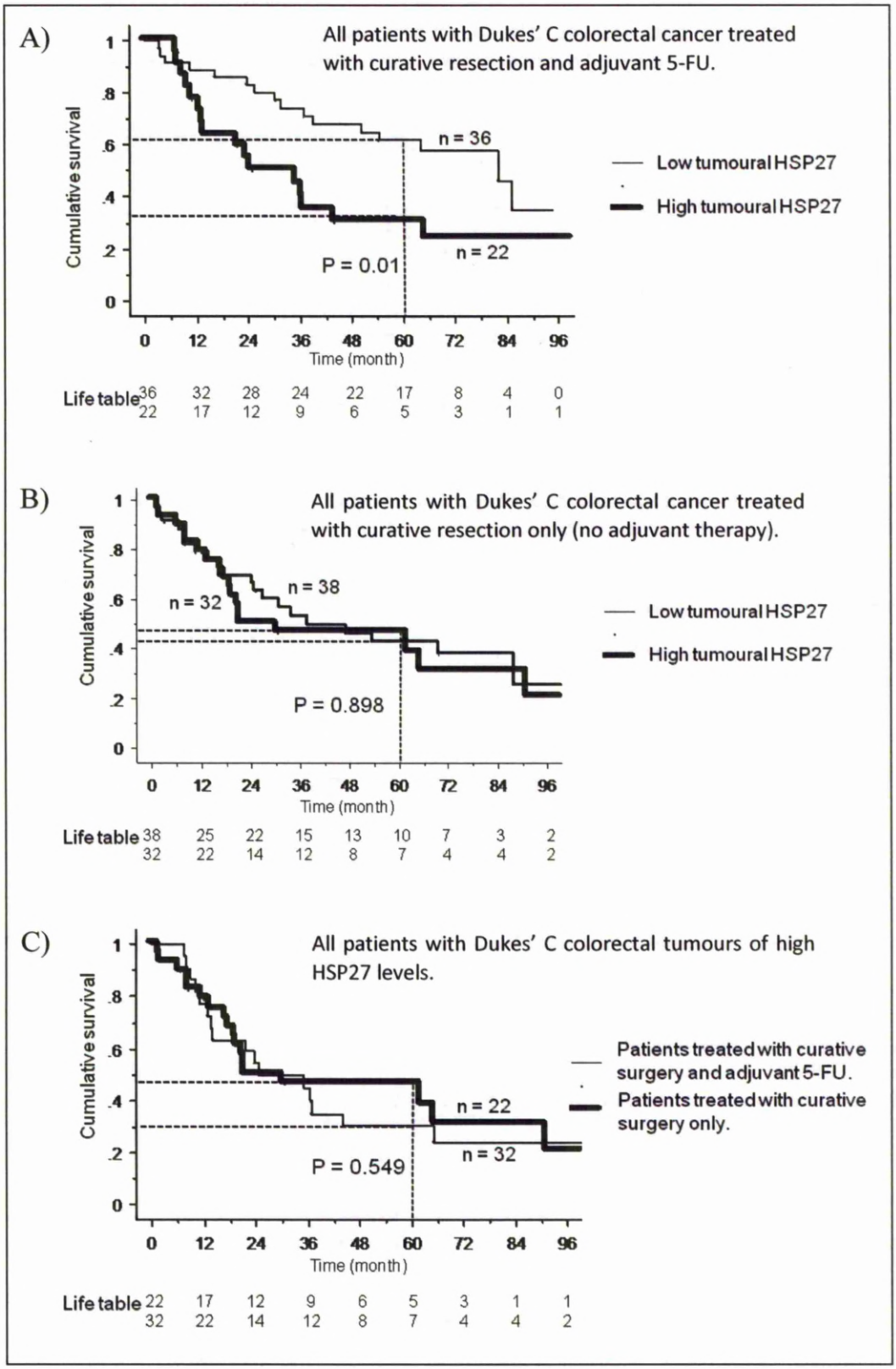
	Dukes' C, Surgery only				Dukes' C, Surgery + adjuvant 5-FU			
	Total, n = 70	**Low HSP27 expression, n = 38**	**High HSP27 expression, n = 32**	***P* value**	**Total, n = 58**	**Low HSP27 expression, n = 36**	**High HSP27 expression, n = 22**	***P* value**
Gender								
Male	33	19	14	0.601‡	38	24	14	0.813‡
Female	37	19	18		20	12	8	
Age (median = 68 [35-90])								
≥ 68	52	27	25	0.500‡	13	6	7	0.179‡
< 68	18	11	7		45	30	15	
Tumour site								
Colon	42	26	16	0.117‡	29	19	10	0.588‡
Rectum	28	12	16		29	17	12	
Tumour Size (median = 50mm)								
≥ 50	39	23	16	0.377‡	32	23	9	0.087‡
< 50	31	15	16		26	13	13	
Degree of differentiation								
Well	1	0	1	0.522‡	0	0	0	>0.999*
Moderate	61	34	27		55	34	21	
Poor	8	4	4		3	2	1	
Resection margin								
Positive	12	6	6	0.743‡	6	4	2	>0.999*
Negative	58	32	26		52	32	20	
T-Stage								
I	1	0	1	0.739‡	2	1	1	0.706‡
II	4	2	2		7	4	3	
III	49	27	22		43	26	17	
IV	16	9	7		6	5	1	
N-Stage								
I	36	20	16	0.826‡	32	21	11	0.535‡
II	34	18	16		26	15	11	
5-year cancer-specific death								
Yes	33	18	15	0.967‡	28	13	15	***0.017‡***
No	37	20	17		30	23	7	

‡ Chi-squared test
* Fisher-exact test

Table 14: Association between HSP27 expression and clinico-pathological features.

5.1.3 HSP27 expression and association with cancer-specific 5-year survival

To further examine the observed difference in cancer-specific death rates, Kaplan-Meier survival analyses were performed on the two cohorts. Among patients treated with surgery followed by adjuvant 5-FU, patients with high tumoural HSP27 had significantly poorer survival ($P = 0.01$, Figure 17A), while HSP27 expression was not associated with outcome in patients treated with surgery only ($P = 0.89$, Figure 17B). Patients with high tumoural HSP27 did not have improved survival following surgery and adjuvant 5-FU compared to patients with high tumoural HSP27 who have received surgical treatment only ($P = 0.54$, Figure 17C). Furthermore, among patients with low tumoural HSP27 expression, there was an observed better survival among patients treated with surgery and adjuvant 5-FU compared to patients treated with surgery only, although this did not reach statistical significant level ($P = 0.087$, Figure 17D). Unfortunately, the relatively small number of patients treated with surgery and adjuvant 5-FU in this study has precluded analysis according to anatomical site (n = 29 each for the colonic and rectal cancer patient group).


A)
All patients with Dukes' C colorectal cancer treated with curative resection and adjuvant 5-FU.
Cumulative survival
1
.8
.6
.4
.2
0
n = 36
n = 22
P = 0.01
Low tumoural HSP27
High tumoural HSP27
0 12 24 36 48 60 72 84 96
Time (month)
Life table 36 32 28 24 22 17 8 4 0
22 17 12 9 6 5 3 1 1
B)
All patients with Dukes' C colorectal cancer treated with curative resection only (no adjuvant therapy).
Cumulative survival
n = 32
n = 38
P = 0.898
Low tumoural HSP27
High tumoural HSP27
0 12 24 36 48 60 72 84 96
Time (month)
Life table 38 25 22 15 13 10 7 3 2
32 22 14 12 8 7 4 4 2
C)
All patients with Dukes' C colorectal tumours of high HSP27 levels.
Cumulative survival
n = 22
n = 32
P = 0.549
Patients treated with curative surgery and adjuvant 5-FU.
Patients treated with curative surgery only.
0 12 24 36 48 60 72 84 96
Time (month)
Life table 22 17 12 9 6 5 3 1 1
32 22 14 12 8 7 4 4 2

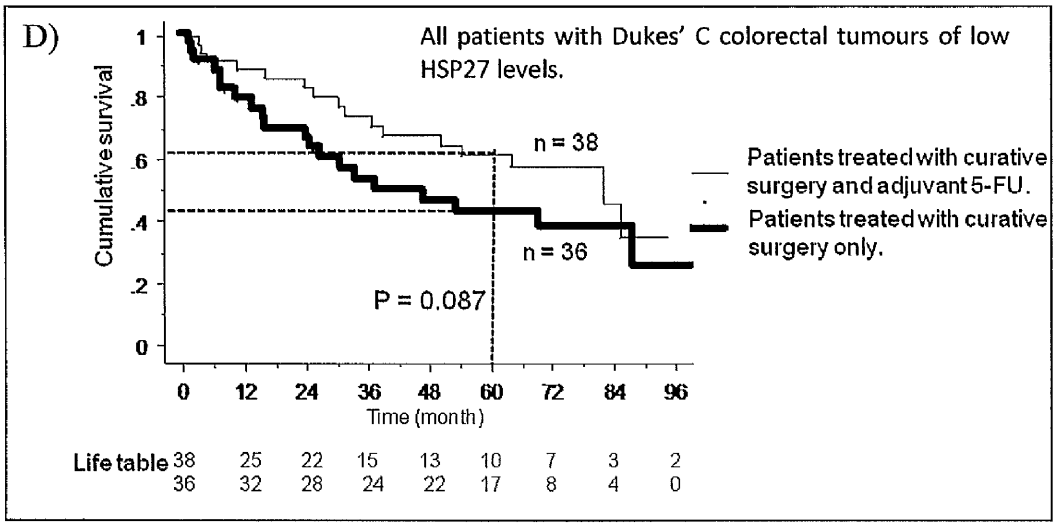


Figure 17: Kaplan-Meier graphical analysis of colorectal cancer patients selected for this study. (A) Patients treated with surgery and adjuvant 5-FU, with high tumoural HSP27 had significantly poorer survival. (B) HSP27 expression did not have survival prognostic value in patients treated with surgery only. (C) Patients with high tumoural HSP27 treated with surgery and adjuvant 5-FU had similar survival compared to patients with high tumoural HSP27 treated with surgery only. (D) In patients with low tumoural HSP27 expression, there was an observed improved survival among patients treated with surgery and adjuvant 5-FU compared to patients treated with surgery only.

5.1.4 Univariate analysis and multivariate Cox proportional hazards regression analysis

Having observed that patients treated with surgery and adjuvant 5-FU, with high tumoural HSP27 expression tumours had poorer survival, univariate and multivariate Cox proportional hazards regression analyses were performed in order to assess whether HSP27 was an independent predictive marker in this cohort of patients. There were 58 patients treated with surgery and adjuvant 5-

FU, divided into tumours with high HSP27 expression (n = 22) and low HSP27 expression (n = 36) (Table 15). Parameters associated with cancer-specific survival on univariate analysis with a P value of < 0.1 were considered significant and included in the multivariate model using a forward stepwise approach [297]. Univariate analysis revealed that cancer cell differentiation, N-stage and high HSP27 expression were associated with poorer survival (Table 15). Subsequent multivariate Cox regression analysis revealed that only high HSP27 expression as an independent predictive marker of poor survival (Hazard Ratio 2.639 [1.233 – 5.647], $P = 0.012$).

Predictors	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Age	0.975 (0.938 – 1.014)	0.204	-	
Gender	1.695 (0.799 – 3.595)	0.168	-	
Tumour site	1.026 (0.489 – 2.153)	0.946	-	
Maximum tumour diameter	0.994 (0.985 – 1.004)	0.215	-	
Differentiation	**3.627 (1.089 – 12.080)**	**0.035**	2.714 (0.768 – 9.588)	0.121
Positive resection margin	1.107 (0.334 – 3.669)	0.867	-	
T stage	0.674 (0.203 – 2.234)	0.518		
N stage	**2.441 (1.135 – 5.247)**	**0.022**	2.144 (0.960 – 4.785)	0.062
High HSP27 expression	**2.574 (1.217 – 5.446)**	**0.013**	**2.639 (1.233 – 5.647)**	**0.012**

Table 15: Univariate and multivariate analyses of patients treated with surgery and adjuvant 5-FU.

5.1.5 HSP27 knockdown

My observation that patients treated with adjuvant 5-FU with high tumoural HSP27 expression had poorer outcome compared with patients with low tumoural HSP27 expression, suggested that high HSP27 levels were predictive of poor response to adjuvant 5-FU. At the time of undertaking this study, there was only one published study examining the effects of HSP27 levels on the response of colon cancer cell lines to 5-FU[290]. This study, however showed that HSP27 overexpression was not accompanied by increased resistance to 5-FU. We therefore sought to examine whether depleting HSP27 expression would enhance efficacy of 5-FU, other chemotherapeutic agents and irradiation on colorectal cancer cells.

HSP27 expression was observed in the four colorectal cancer cell lines, HCT116, SW480, HRT18 and SW837 (Figure 18A), with HRT18 cells exhibiting the lowest level in the non-induced state. In the cases of SW480, HCT116 and HRT18 cells, significant HSP27 depletion was apparent at 3 days post incubation with HSP27-targeting siRNA incubation, a time point when cells were collected for further experiments. HSP27 levels remained low at 7 days post-treatment with HSP27-targetting siRNA, compared to controls, although a small degree of recovery of HSP27 was evident in SW480 cells (Figure 18B). In the case of SW837 cells, HSP27 depletion was not apparent at 3 days (data not shown) but the depletion of this protein was observed 4 days post siRNA incubation, and was still low at 11 days post-knockdown (Figure 18C).

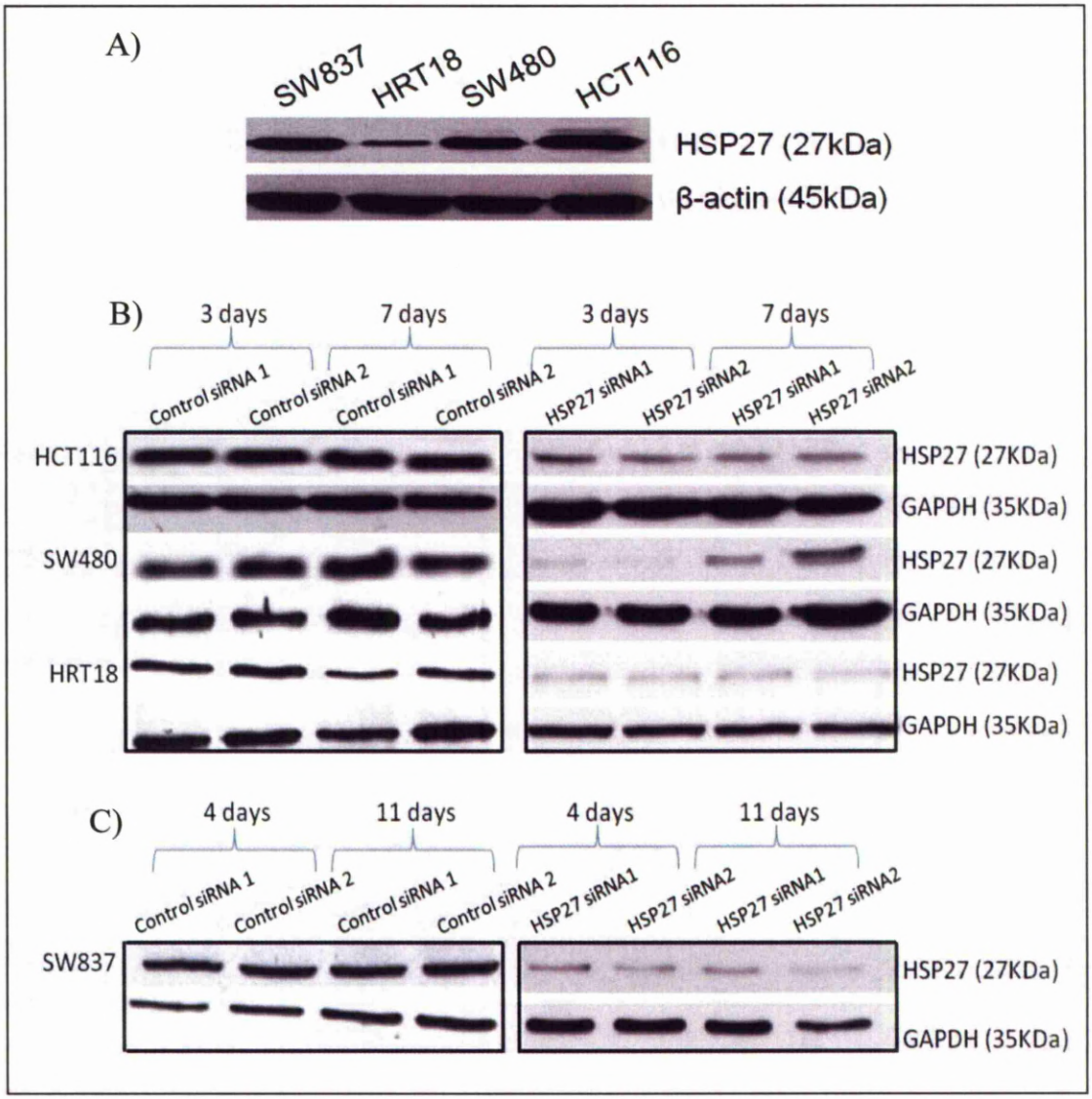
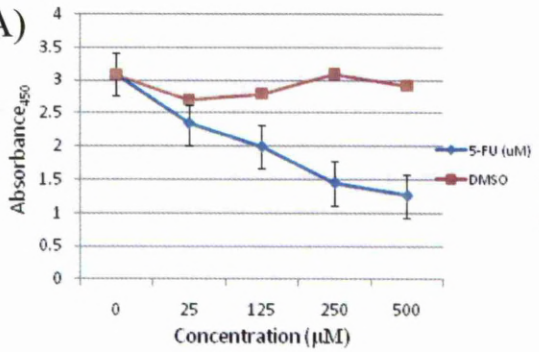


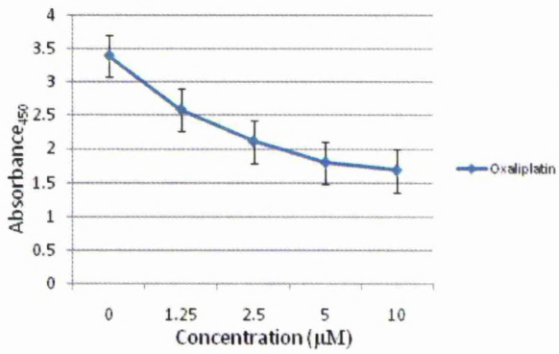
Figure 18: Western blots illustrating transient HSP27 depletion using siRNAs, and for comparison, control siRNAs, on colorectal cancer cell lines. (A) HSP27 expression was observed in the cell lines used in this study. (B) HSP27 depletion in SW480, HCT116 and HRT18 cells. (C) HSP27 depletion in SW837 cells.

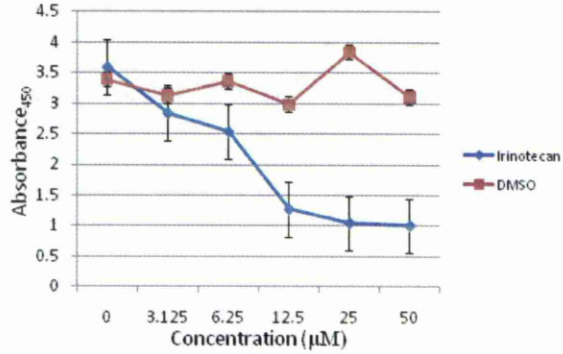
5.1.6 HSP27 depletion enhanced cell apoptosis in response to chemo- or irradiation

Using MTS assay, the half maximal inhibitory concentrations (IC_{50}) of three chemotherapy agents - 5-FU, irinotecan, oxaliplatin, and of gamma irradiation on HCT116, SW480, HRT18 and SW837 cells were measured (Figure 19). For ease of comparison, all of the IC_{50}s from Figure 19 were tabulated (Table 16). Based on these data, the rectal cancer cells, SW837, exhibited the highest IC_{50} values and therefore the most resistance to 5-FU, oxaliplatin and gamma irradiation (1,000 μM, 150 μM and 15 Gy respectively, Figure 19D & E, Table 16). The other rectal cancer cells, HRT18, were the most resistant to irinotecan (75 μM), but at the same time, the most sensitive to 5-FU and oxaliplatin (100 μM and 1.56 μM respectively) compared to other cancer cell lines in this study (Figure 19C). The colon cell line HCT116 showed the lowest IC_{50} value and therefore was most sensitive to 5-FU (Figure 19A). These IC_{50}s were then used in apoptosis assays to examine the effects of HSP27 depletion on cellular sensitivity to chemotherapy and radiotherapy.

A)

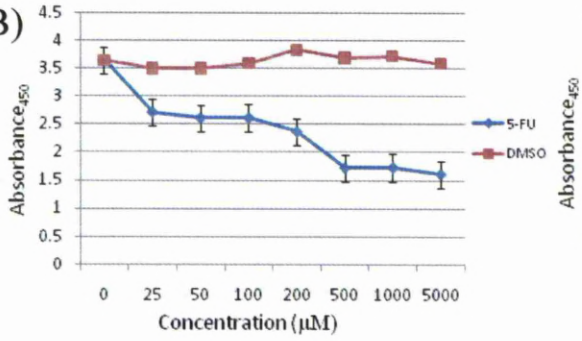


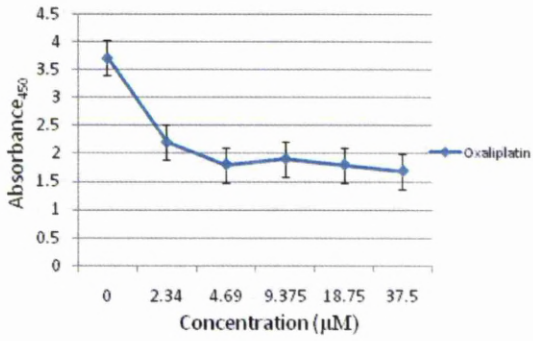


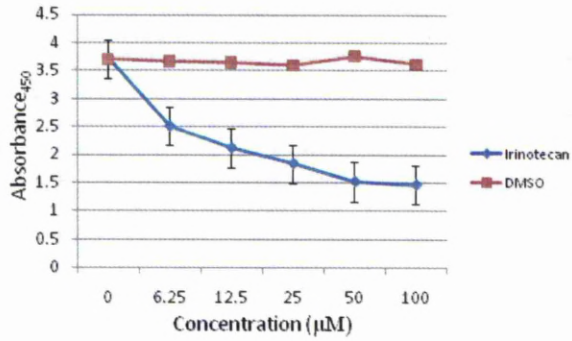


IC_{50} of chemotherapy agents for HCT116 (4 days of incubation):
5-FU: 250 μM
Oxaliplatin: 5 μM
Irinotecan: 12.5 μM

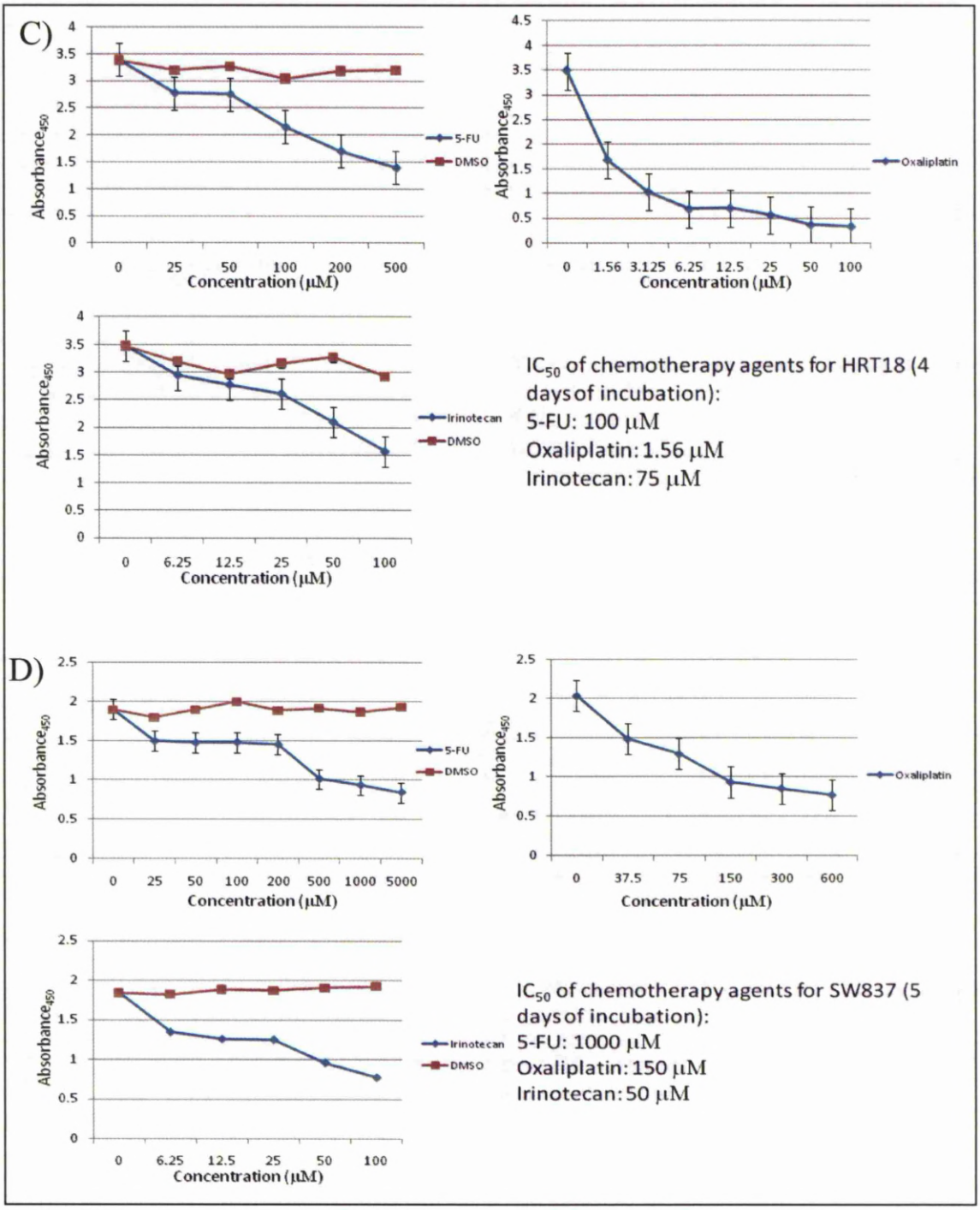
B)







IC_{50} of chemotherapy agents for SW480 (4 days of incubation):
5-FU: 500 μM
Oxaliplatin: 4.69 μM
Irinotecan: 25 μM


C)
Absorbance450
Concentration (μM)
0 25 50 100 200 500
5-FU
DMSO
0 1.56 3.125 6.25 12.5 25 50 100
Oxaliplatin
0 6.25 12.5 25 50 100
Irinotecan
IC50 of chemotherapy agents for HRT18 (4 days of incubation):
5-FU: 100 μM
Oxaliplatin: 1.56 μM
Irinotecan: 75 μM
D)
0 25 50 100 200 500 1000 5000
0 37.5 75 150 300 600
0 6.25 12.5 25 50 100
IC50 of chemotherapy agents for SW837 (5 days of incubation):
5-FU: 1000 μM
Oxaliplatin: 150 μM
Irinotecan: 50 μM

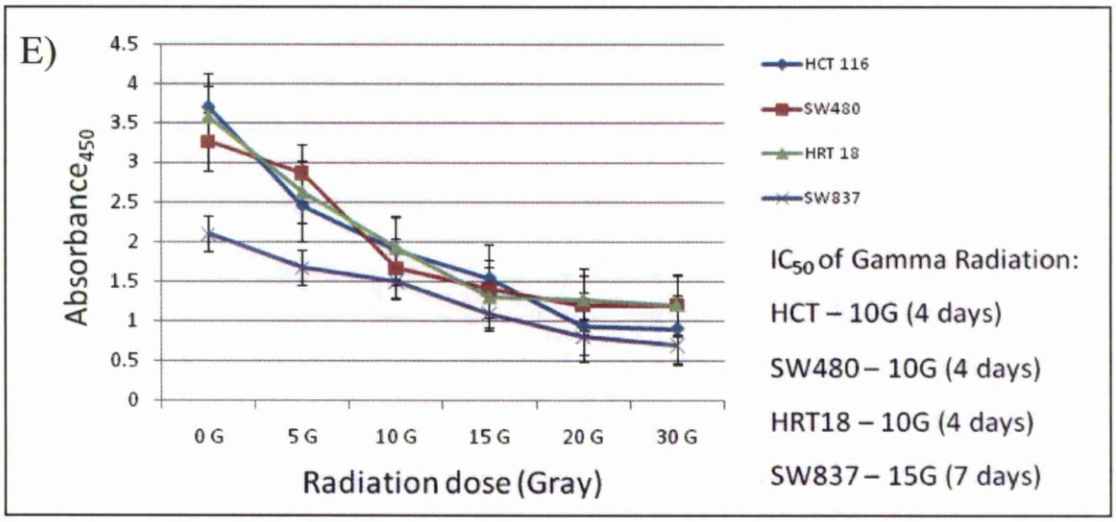


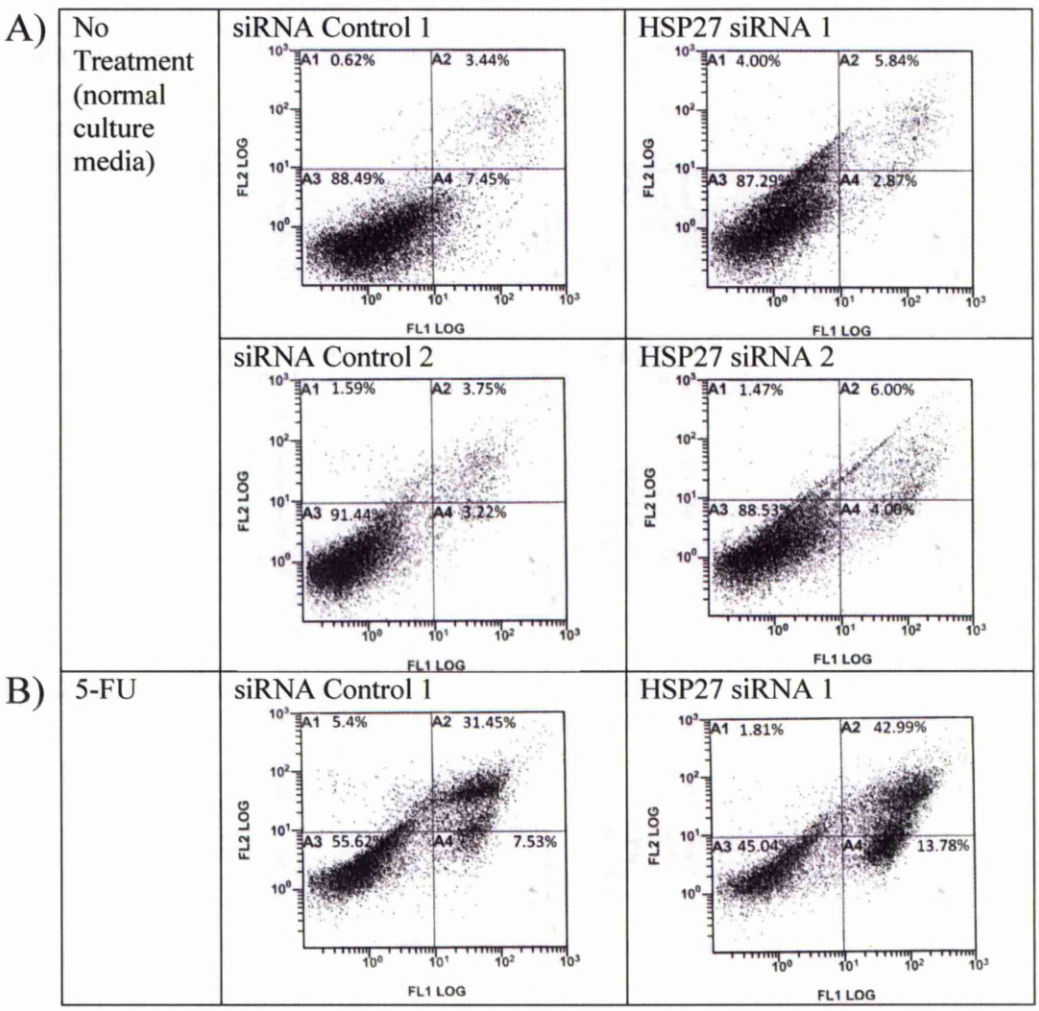
Figure 19: IC_{50} of chemo and radiotherapy for colorectal cancer cells lines. Cells were harvested and 3,000 cells/well were plated for 24 hr prior to addition of variable concentrations of chemotherapy or gamma irradiation. HCT116, SW480 and HRT18 cells were incubated for 4 days following treatment with chemotherapeutic agents and gamma irradiation, whilst SW837 cells were incubated for 5 days following addition of chemotherapeutic agents and 7 days after treatment with gamma irradiation. Using MTS assays, IC_{50} of 5-FU, irinotecan and oxaliplatin for A) HCT116, B) SW480, C) HRT18, D) SW837 cells and gamma radiation for D) 4 cell lines were determined. The results are representative of at least 2 independent experiments with five wells utilized for each treatment.

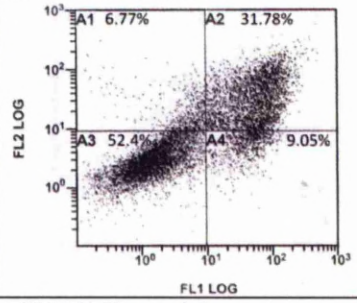
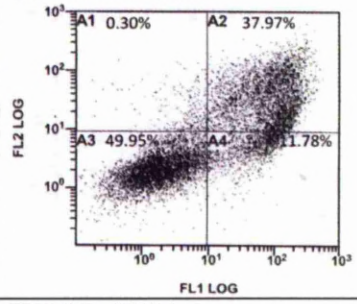
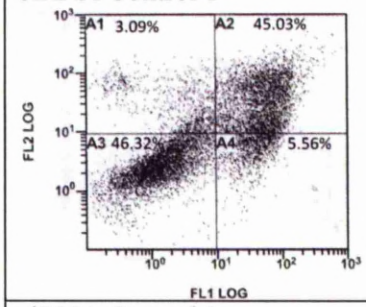
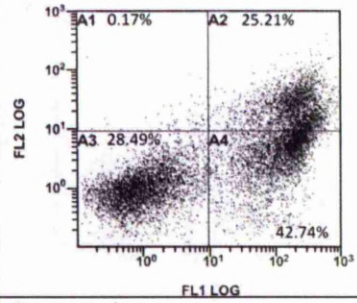
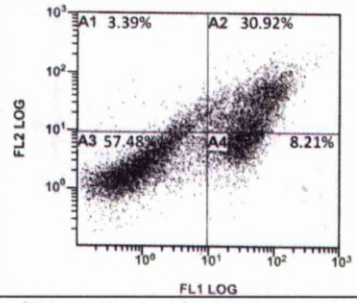
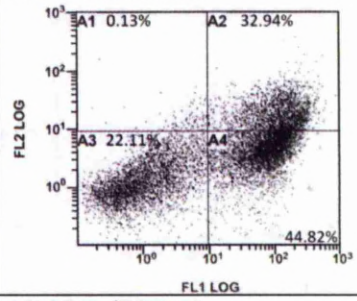
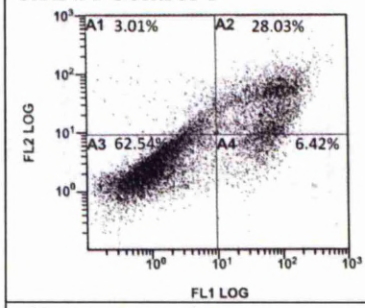
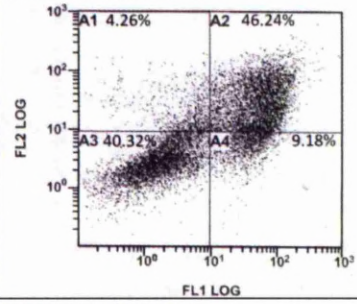
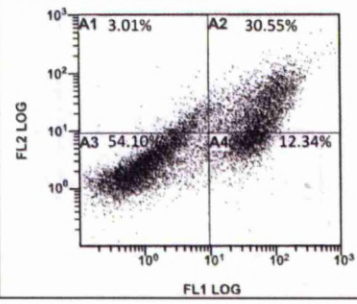
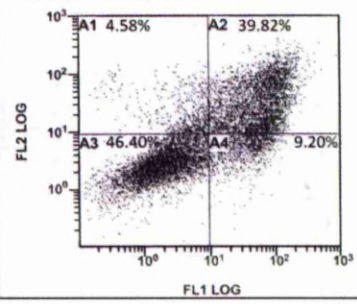
IC_{50} value / Cell line	5-FU (μM)	Irinotecan (μM)	Oxaliplatin (μM)	Gamma irradiation (Gray)
HCT116	250	12.5	5	10
SW480	500	25	4.69	10
HRT18	100	75	1.56	10
SW837	1000	50	150	15

Table 16: Tabulated IC_{50} values of chemotherapeutic agents and gamma irradiation for colorectal cancer cell lines used in this study.

When HCT116 cells were subjected to siRNA transfection, HSP27 depletion alone did not result in a significant increase in cell apoptosis (quadrants A2 + A4, compared to cells treated with control siRNAs) (shown as flow cytometry two-colour fluorescence dot plot in Figure 20A, and the same of average of repeated experiments shown in graphical form in Figure 21A). Treatment of HSP27-depleted HCT116 cells with 5-FU (Figure 20B), oxaliplatin (Figure 20C), irinotecan (Figure 20D) and gamma radiation (Figure 20E), was accompanied in every case by a significant increase in apoptotic cells compared to the control siRNA treated cells ($P < 0.05$, Figure 21A). This significant increase in cell chemo-radio sensitivity following HSP27 depletion was also consistently shown in SW480, HRT18 and SW837 cells (Figure 21B, C & D respectively). For HSP27-depleted cells, there was a 10-20 % increase in the apoptotic population compared with non-HSP27-treated cells upon treatment with 5-FU, oxaliplatin or irinotecan (Figure 21). This magnitude of increase in cell apoptosis was smaller upon treatment with gamma irradiation, ~10 %. Furthermore, the most 5-FU-, oxaliplatin-, and irradiation-resistant cells, SW837

showed a similar increase in cell response to these treatments following HSP27 depletion compared with other cell lines. There was however, no difference between colon (HCT116 and SW480) and rectal cancer cells (HRT18 and SW837) in the extent of response to chemo- or radiation upon HSP27 depletion. The depletion of HSP27 expression following treatment with HS27-targetting siRNAs in every experiment was confirmed by western blotting.



		siRNA Control 2	HSP27 siRNA 2
			
C)	Irinotecan	siRNA Control 1	HSP27 siRNA 1
			
		siRNA Control 2	HSP27 siRNA 2
			
D)	Oxaliplatin	siRNA Control 1	HSP27 siRNA 1
			
		siRNA Control 2	HSP27 siRNA 2
			

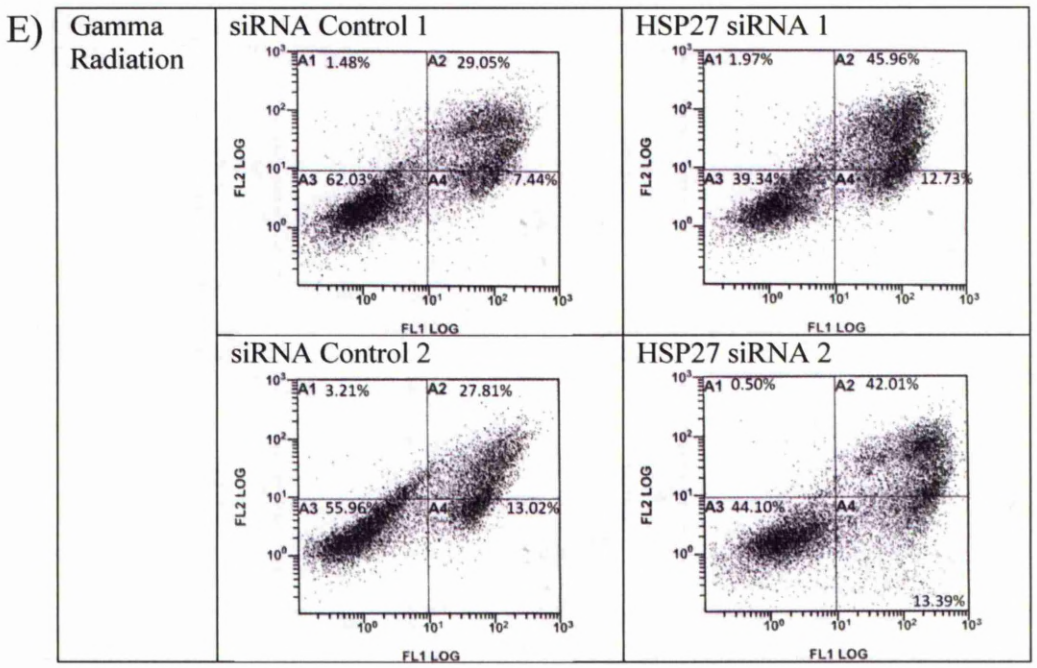


Figure 20: Flow cytometry two-colour fluorescence dot plot of HCT116 cells labelled with annexin-V and propidium iodide. Three days following transfection with HSP27-targeting and control siRNAs, HCT116 cells were treated with 4 days of A) normal culture media, B) 5-FU, C) Oxaliplatin, D) Irinotecan, and E) Gamma irradiation, followed by cell collection for annexin-V/propidium iodide staining and flow cytometry analysis. The x-axis represents annexin-V-related fluorescence (FL1 log) and the y-axis represents propidium iodide-related fluorescence (FL2 log). The percentages of cells in quadrants are representative of cell debris (quadrant A1), late apoptotic cells (quadrant A2), viable cells (quadrant A3), and early apoptotic cells (quadrant A4). Cell apoptosis was calculated by adding percentages in quadrants A2 and A4. The results are representative of at least 2 independent experiments.

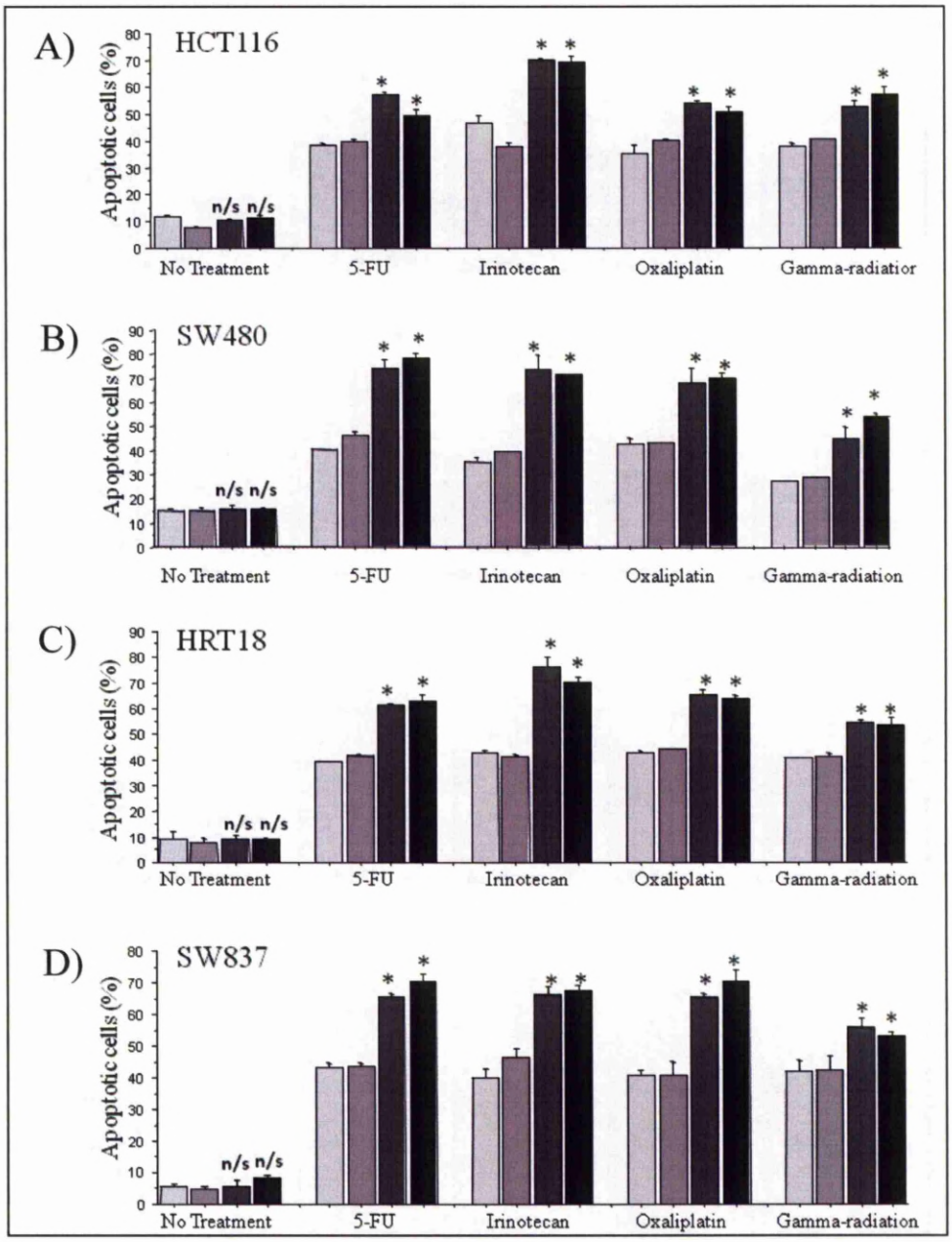


Figure 21: The proportion of apoptotic cells in response to chemo-radiotherapy following HSP27 depletion. Upon HSP27 depletion, the proportion of apoptotic cells was significantly increased in response to 5-FU, irinotecan, oxaliplatin and gamma-radiation in A) HCT116 cells, B) SW480 cells, C) HRT18 cells, and D) SW837 cells compared to control siRNA treated cells. The results are representative of at least 2 independent experiments. * = $P < 0.05$, n/s = non-significant.

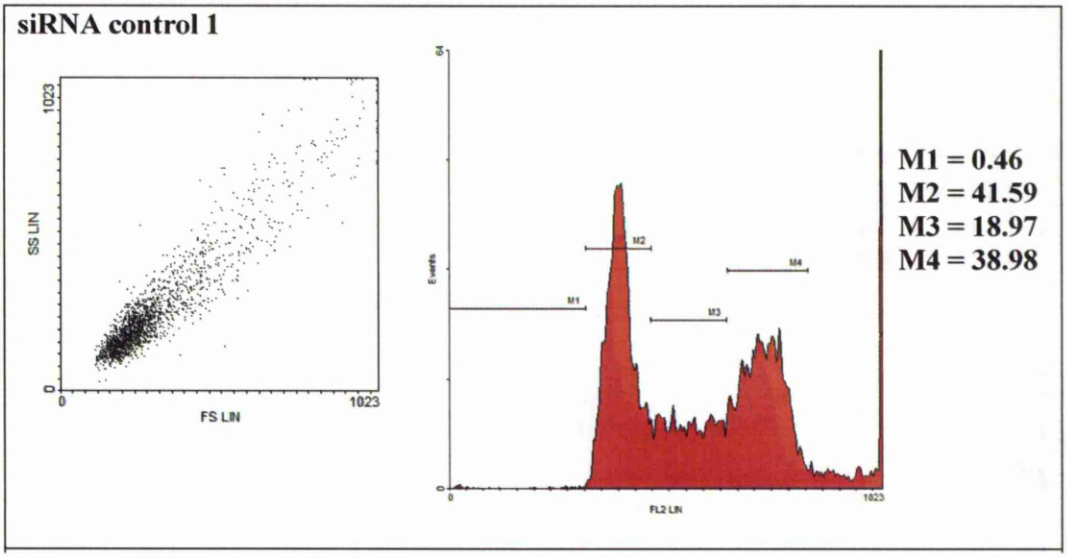
5.1.7 Cell cycle analysis, cell proliferation and cell migration

Cell cycle analysis was performed on HCT116 (Figure 22) and HRT18 (Figure 23) cells following HSP27 depletion. In HCT116 cells, HSP27 depletion resulted in a significantly larger proportion of cells in G_0/G_1 phase compared to the control siRNA treated cells ($P < 0.05$, Figure 22B), with a corresponding lower proportion of cells in S phase and G_2/M phase, although this did not reach statistical significance. These changes in the cell cycle profile following HSP27 depletion were more pronounced in HRT18 cells, where HSP27 depletion resulted in a significant G1 block, accompanied by a considerably lower proportion of cells in S phase and G_2/M phase compared to controls ($P < 0.05$, Figure 23B).

HCT116

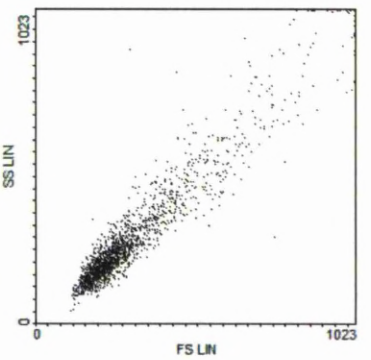
Dot Plot ***Histogram*** ***Stats (%)***

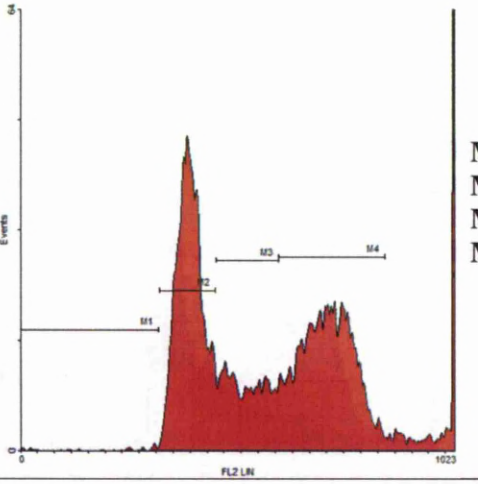
Ai) **siRNA control 1**



Aii)

siRNA control 2

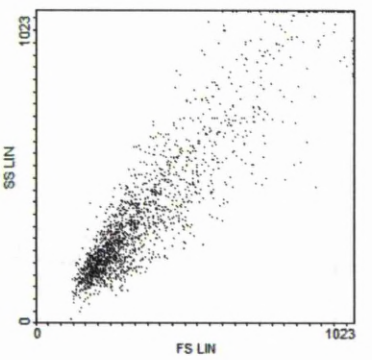


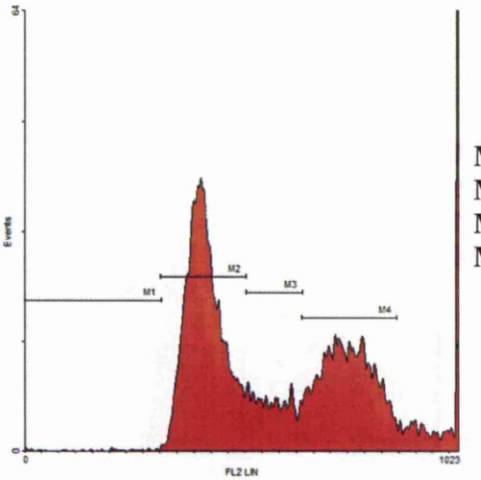


M1 = 0.52
M2 = 39.54
M3 = 17.71
M4 = 42.23

Aiii)

HSP27 siRNA 1

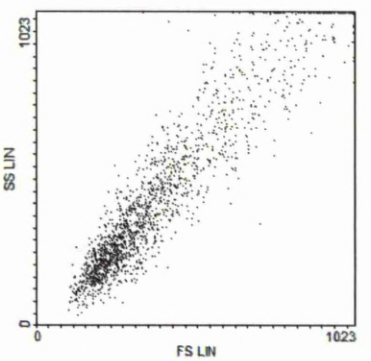


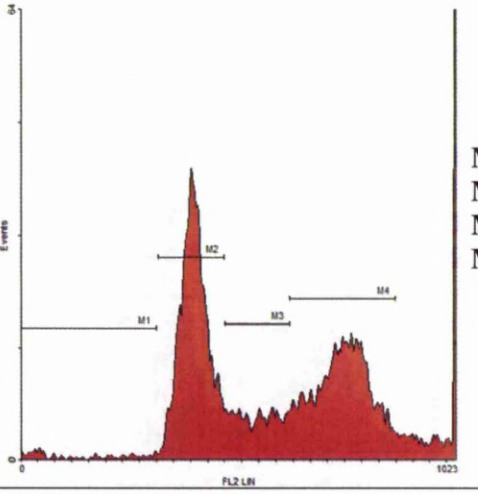


M1 = 0.74
M2 = 49.03
M3 = 13.48
M4 = 36.75

Aiv)

HSP27 siRNA 2





M1 = 2.70
M2 = 47.21
M3 = 13.98
M4 = 36.11

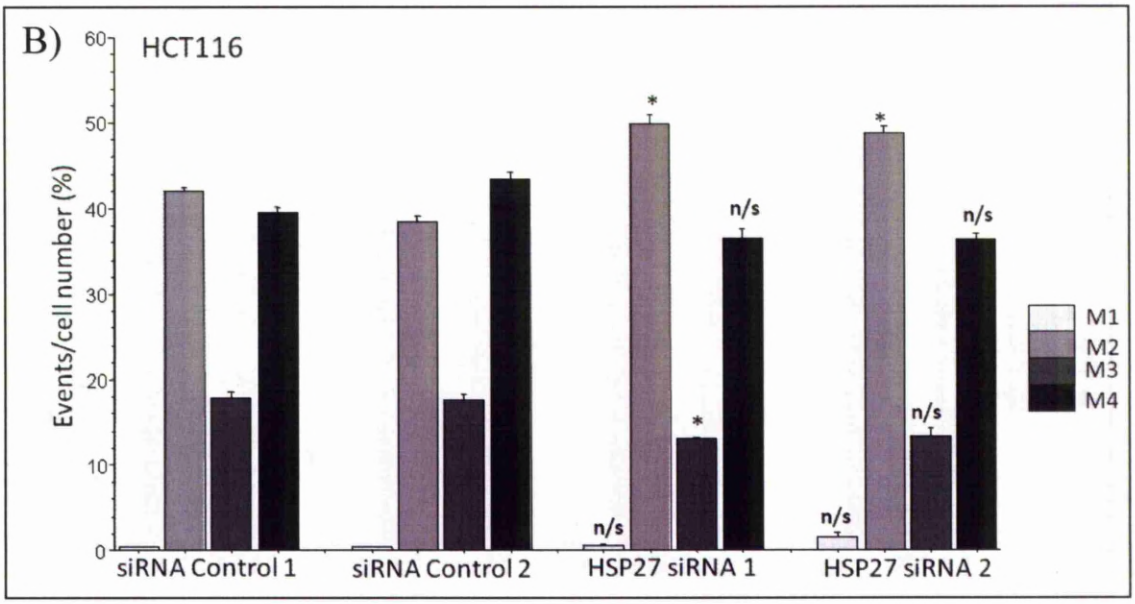
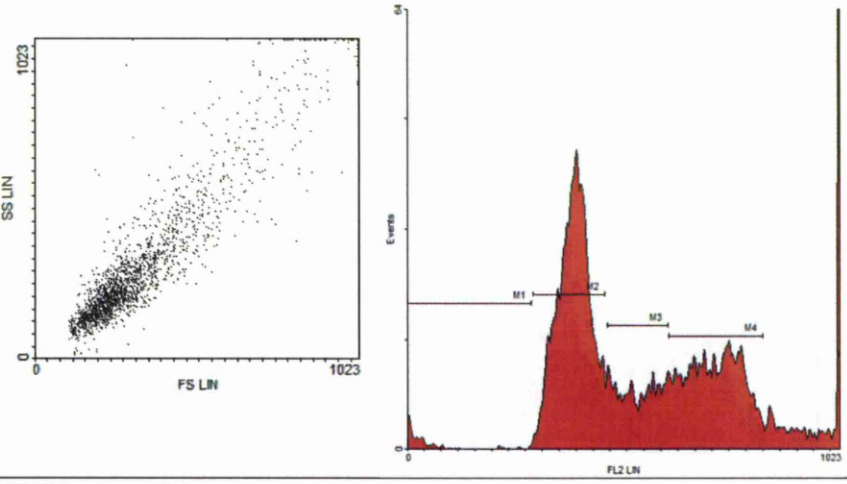


Figure 22: Cell cycle profiles of HCT116 cells following HSP27 depletion. HCT 116 cells were harvested for cell cycle analysis following 3 days of incubation with siRNAs. The resulting cytograms are shown for cells treated with Ai) siRNA control 1, Aii) siRNA control 2, Aiii) HSP27-targeting siRNA 1, and Aiv) HSP27-targetting siRNA 2, while B) shows the percentages of cells in various stages of the cell cycle representative of two independent experiments. M1 denotes Sub-G_0 phase cells, indicative of fragmented DNA dying/dead cells. M2 denotes G_0/G_1 phase cells, which indicates a period in the cell cycle where cells exist in a quiescent state. M3 denotes S phase cells, indicative of synthesis phase, when DNA synthesis or replication occurs. M4 denotes G_2/M phase, when cells undergo rapid growth and mitosis. The results are representative of at least 2 independent experiments. * = $P < 0.05$, n/s = non-significant.

HRT18

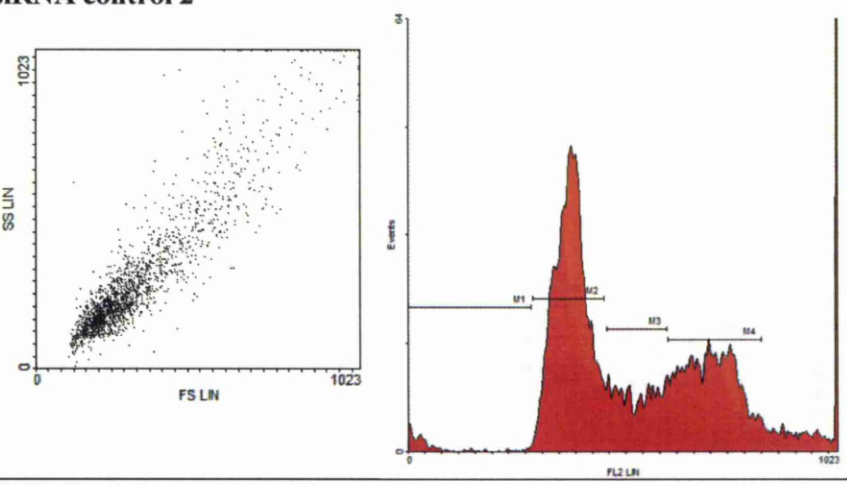
Dot Plot *Histogram* *Stats (%)*

Ai) **siRNA control 1**



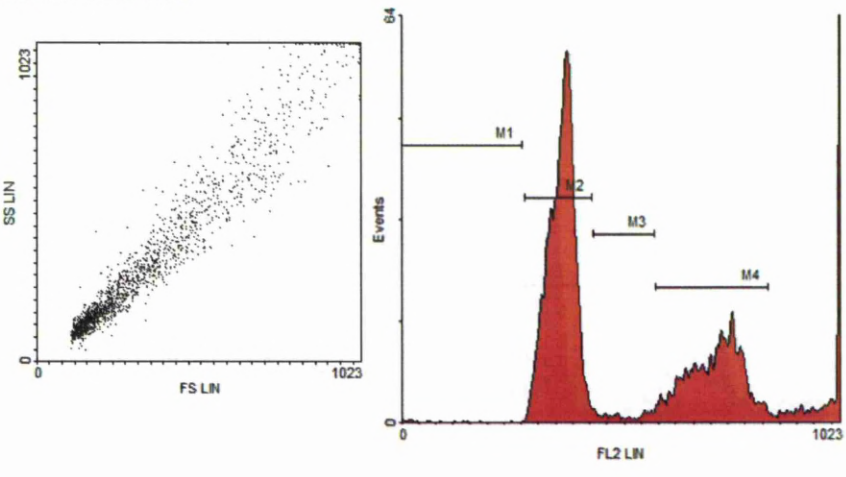
M1 = 1.78
M2 = 48.87
M3 = 16.28
M4 = 33.07

Aii) **siRNA control 2**



M1 = 2.15
M2 = 50.44
M3 = 15.21
M4 = 32.20

Aiii) **HSP27 siRNA 1**



M1 = 0.82
M2 = 73.08
M3 = 3.26
M4 = 22.84

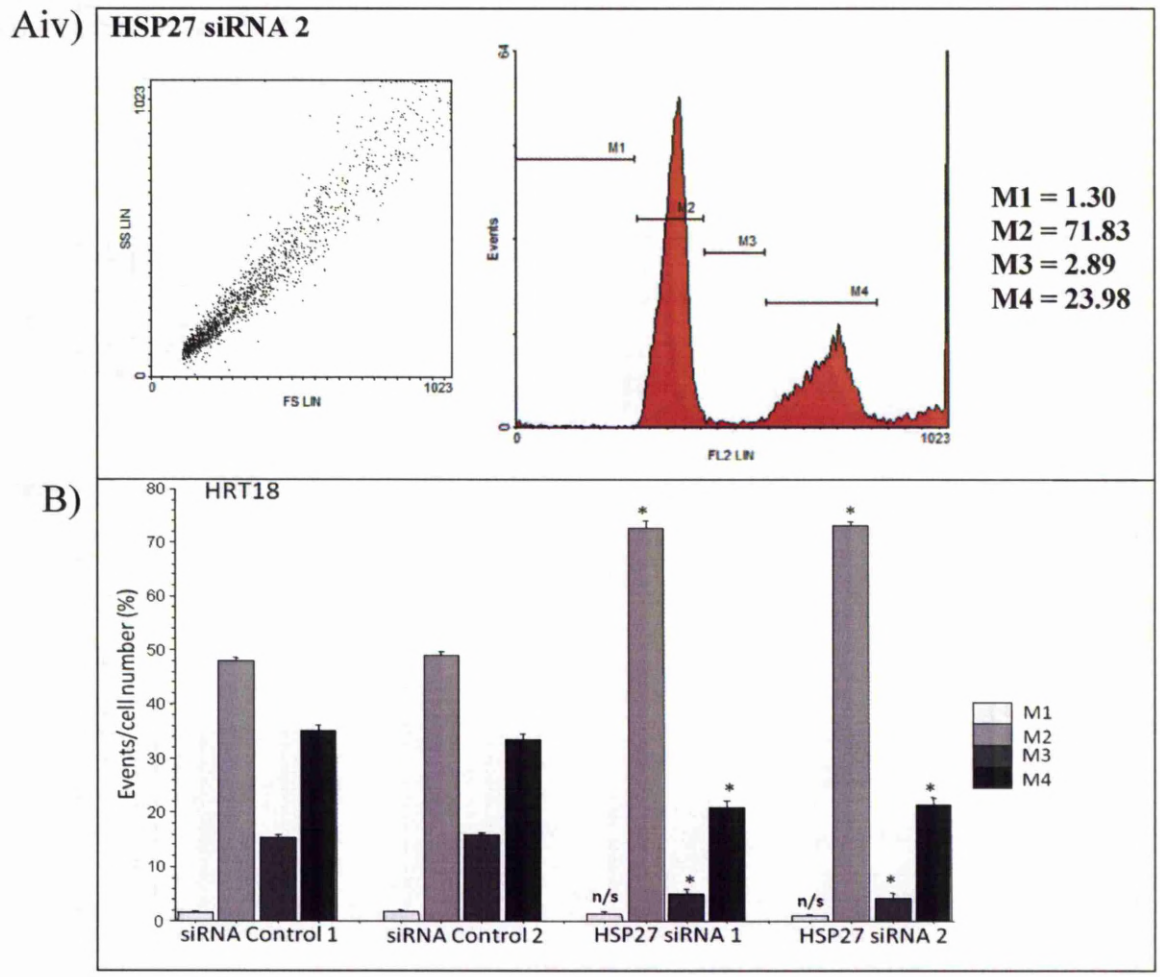


Figure 23: Cell cycle profiles of HRT18 cells following HSP27 depletion. HRT18 cells were harvested for cell cycle analysis following 3 days of incubation with siRNAs. The resulting cytograms are shown for cells treated with Ai) siRNA control 1, Aii) siRNA control 2, Aiii) HSP27-targeting siRNA 1, and Aiv) HSP27-targetting siRNA 2, while B) shows the percentages of cells in various stages of the cell cycle representative of two independent experiments. M1 denotes Sub-G_0 phase cells, indicative of fragmented DNA dying/dead cells. M2 denotes G_0/G_1 phase cells, which indicates a period in the cell cycle where cells exist in a quiescent state. M3 denotes S phase cells, indicative of synthesis phase, when DNA synthesis or replication occurs. M4 denotes G_2/M phase cells, when cells undergo rapid growth and mitosis. The results are representative of at least 2 independent experiments. * = $P < 0.05$, n/s = non-significant.

Along with these changes in cell cycle profiles, HSP27 depletion was consistently accompanied by retardation in cell proliferation in HCT116, SW480, HRT18 and SW837 cell lines (Figure 24A, B, C & D). In addition, *in vitro* cell motility assays showed that there was significant reduction in cell migration in response to 10 % FBS placed in the lower motility chamber, demonstrated by HCT116, SW480 and SW837 cells ($P < 0.05$, Figure 25), although this effect was not observed in the less motile cells, HRT18 (Figure 25).

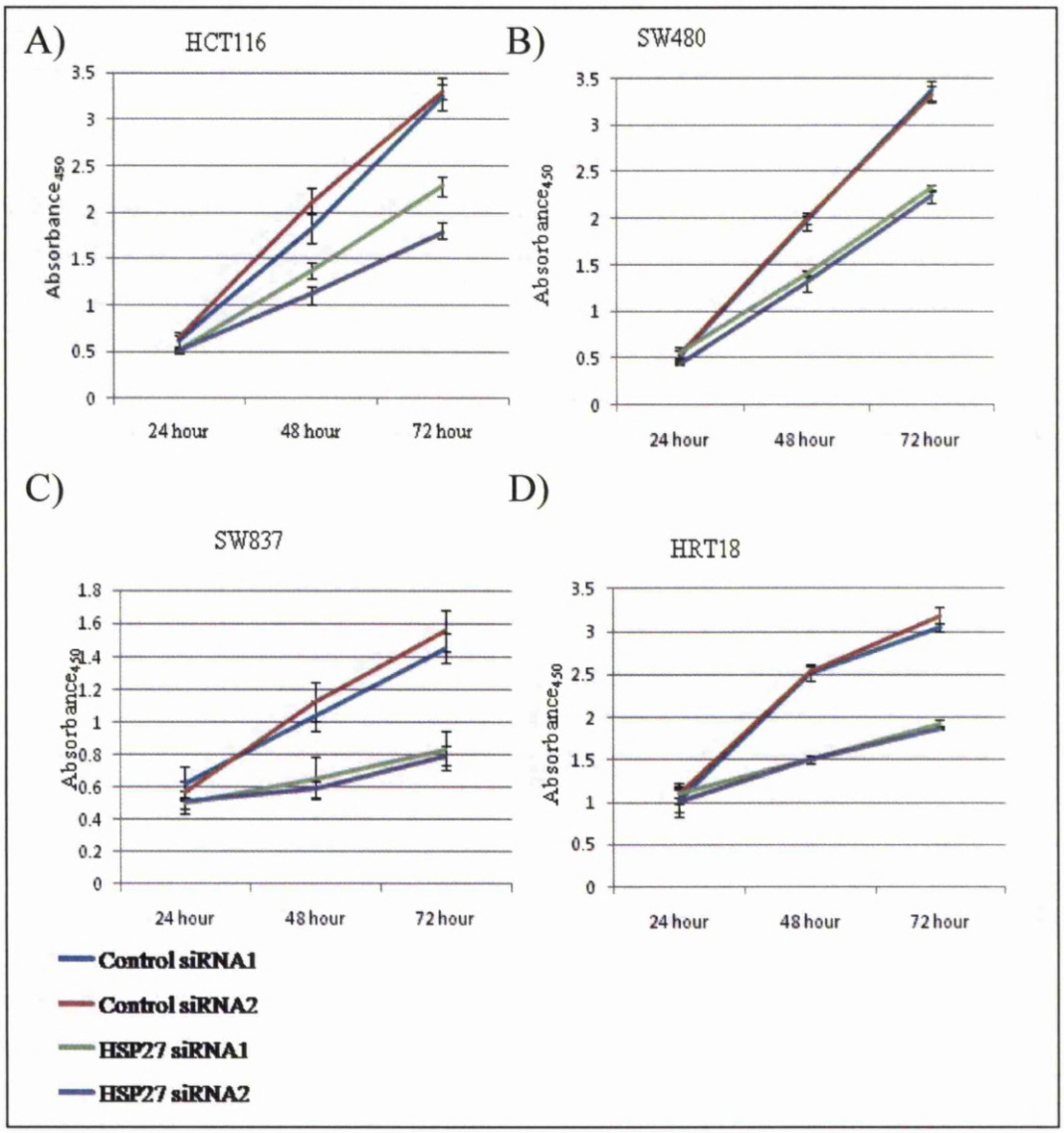


Figure 24: Cell proliferation analysis following HSP27 depletion. Following HSP27 depletion experiments (3 days for HCT116, SW480 and HRT18 cells, and 4 days for SW837 cells following treatment with HSP27 targeting siRNAs or non-targeting controls) , cells were collected and plated into 96-well plate (3,000 cells/well) and incubated for 24, 48 and 72 hours when MTS assays were performed on these time point. MTS readings at absorbance of 450 nm of indicated cells following HSP27 depletion A) HCT116, B) SW480, C) SW837, D) HRT18. The results are representative of at least 2 independent experiments with five wells utilized for cells treated with each siRNAs.

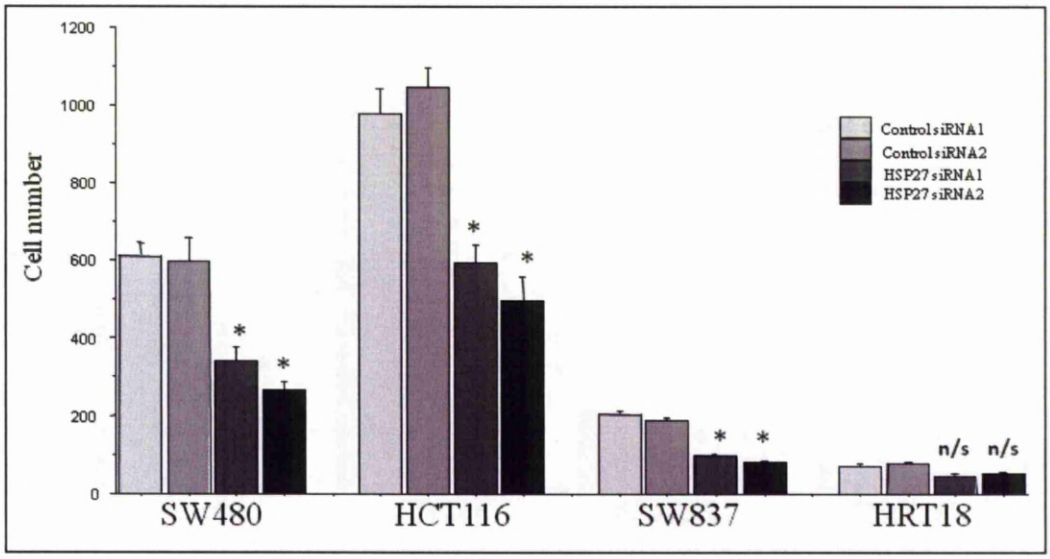


Figure 25: Cell motility following HSP27 depletion. Cells were collected following HSP27 depletion experiment and plated into a modified Boyden chamber, followed by 18 hours incubation before the inserts were stained and cells were counted. The results are representative of at least 2 independent experiments. * = $P < 0.05$, n/s = non-significant.

5.2 Discussion

Following surgical resection, the 5-year survival for node-positive, Dukes' C cancer is only 30-40 %[56]. Hence, patients in this poor prognostic group are routinely considered for adjuvant therapy. The mainstay of adjuvant chemotherapy in colorectal cancer is 6 months administration of 5-fluorouracil (5-FU), which confers a 30 % proportional reduction in risk of death, equivalent to an additional survival gain of 10-15 %[298]. A large proportion of patients receiving 5-FU unfortunately will not actually derive benefit from it, either because surgery alone would be curative or because the disease will ultimately relapse despite the additional treatment.

Can the current adjuvant treatments be improved? They could be improved if they were better tolerated, more effective, or targeted more closely to the patients who would actually benefit/respond. At present, there is no molecular marker that is sufficiently accurate to be used in the clinical practice as a predictive factor for response to adjuvant chemo-radiotherapy in colorectal cancer.

The relatively large Liverpool colorectal cancer patient cohort (n = 404), which had previously undergone characterisation for HSP27 expression[138], has afforded smaller subgroup analysis of Dukes' C cancer patients treated with adjuvant 5-FU (n = 58).

Our observation of an association between high tumoural HSP27 and poor cancer-specific 5-year survival among Dukes' C cancer patients who had undergone surgery and had received adjuvant 5-FU suggests that high HSP27 confers resistance to 5-FU and therefore leads to early recurrence and poor

survival. Moreover, our finding that for patients with low tumoural HSP27, treatment with adjuvant 5-FU was accompanied by an improvement in survival compared to patients with low tumoural HSP27 treated with surgery only is consistent with a role for HSP27protecting against the effects of treatment.

Furthermore, our finding that patients with high tumoural HSP27 who received adjuvant 5-FU did not have an improved outcome compared to patients who did not receive adjuvant 5-FU, suggests that high HSP27 may be predictive of patients who will not derive benefit from 5-FU. Unfortunately, the number of patients in this study was too small to allow us to clarify whether this observation is true in both colon and rectal cancer patients. Moreover, we did not perform genomic analysis on the tumours, such as *KRAS* mutational status, 18q loss of heterozygosity or MSI, which could potentially affect patient outcomes.

Our findings coincide with a smaller cohort analysis (n = 20) by Choi and colleagues[279], which found that HSP27 expression levels of colorectal tumour specimens from irinotecan-non-responding patients were significantly higher compared to those of irinotecan-responding patients. This suggested that HSP27 is associated with irinotecan resistance in human colorectal cancer. Contrary to the findings by Choi and colleagues[279] and our analyses, Rau and colleauges[278] found no correlation between differential HSP27 expression and the level of clinical response in patients treated with pre-operative chemotherapy, radiotherapy or hyperthermia for locally advanced rectal cancer (n= 23).

Based on the perceived clinical relevance of HSP27 in chemo- or radiation resistance in colorectal and other cancers, the consequences of depleting HSP27 levels on cell sensitivity to chemotherapy and radiotherapy has been intensely

studied. For many cancer cell lines grown in culture, siRNA or antisense oligonucleotide-mediated depletion of HSP27 has been shown to enhance apoptosis in response to chemotoxic treatments. Specific examples include enhanced apoptosis following HSP27 depletion in response to vincristine and adriamycin in human gastric cancer cells[282]; paclitaxel in prostate[281] and bladder[283] cancer cells; cisplatin in cervical cancer cells[286]; gemcitabine in pancreatic cancer cells[275]; irinotecan[279] and doxorubicin[290] in colonic cancer cells and gamma-irradiation in several cancer cell lines[289]. However, of particular relevance to our observation that high tumoural HSP27 was associated with poor cancer-specific 5-year survival among patients who had received adjuvant 5-FU, we were aware that Garrido and colleagues[290] found no changes in cell sensitivity to 5-FU in their stably transfected colonic cells with HSP27 cDNA sense. Moreover, there was no published study reporting on the effects of HSP27 levels on chemosensitivity of rectal cancer cell lines. This prompted us to examine whether we could, in a variety of cell lines, determine the relationship between HSP27 and sensitivity to 5-FU and other chemotoxic treatments. We showed that both colon and rectal cancer cell apoptosis was significantly enhanced in response to 5-FU, irinotecan, oxaliplatin and gamma irradiation following transient HSP27 depletion. Our data are consistent with a recent study, which described a colonic cancer cell line transiently transfected with a HSP27-expression vector[299]. The work showed that resistance to 5-FU was significantly enhanced following the overexpression of the protein, and that sensitivity to 5-FU was increased following HSP27 down-regulation.

Cell proliferation and migration are prerequisites for cancer growth, invasion and metastasis. We demonstrated that HSP27 depletion resulted in suppression

of cell proliferation without an increase in cell apoptosis, while Rocchi and colleagues[281, 284, 285] and Zoubeidi and co-workers[287] observed cell growth suppression which could be attributed to an increase in cell apoptosis. Our findings can be explained by the cell cycle analysis, that following HSP27 depletion, the fraction of cells in G_0/G_1 phase was significantly higher, along with lower fraction of cells in S phase and G_2/M phase compared to the controls, suggesting cell cycle arrest and therefore retardation in cell growth. It is however interesting to note, that although HSP27 depletion resulted in arrest of the cell cycle in G_1, it was nonetheless accompanied by an increased in cell apoptosis in response to 5-FU, despite the fact that 5-FU is an S-phase-specific agent. It is possible that the effects of metabolites of 5-FU which also result in DNA and RNA damage (in a non-S-phase dependent manner), may have contributed to the enhanced apoptosis observed[300]. With the lack of HSP27 in inhibiting apoptotic pathways and maintaining cell repair mechanisms, the presence of DNA and RNA damage will ultimately direct cells to commit apoptosis. Together with our observation, *in vivo* studies on mice bearing prostate[284] and bladder[283, 288] cancers confirmed that treatment with HSP27 anti-sense oligonucleotide inhibited tumour growth and decreased tumour volume.

We demonstrated that cell motility was also shown to be significantly reduced following HSP27 depletion. Consistent with our findings, Shin and colleagues[301] reported that HSP27 depletion in breast cancer cells inhibited cell motility and invasion. The group also showed that this is achieved via blocking of protein kinase C-dependent phosphorylation of HSP27. HSP27 has been recognised as a potent regulator of cytoskeletal dynamics in actin microfilaments and stabilises

F-actin microfilaments[247]. Hence, HSP27 depletion results in cytoskeletal instability which leads to impediment in cell motility.

In summary, we have shown for the first time, the clinical relevance of high HSP27 as a predictive marker of poor response to adjuvant 5-FU in patients with resected Dukes C colorectal cancer. This, coupled with our data demonstrating that targeting HSP27 could enhance colorectal cancer cell sensitivity to chemotherapy and radiotherapy suggest a potential new avenue in the management of colorectal cancer.

CHAPTER 6:
CONCLUSION

The roles of endogenous DAMPs in sustaining cancer cell proliferation, motility, migration, interaction with stromal cells, endurance from external insults (i.e. chemo-radiotherapy) are now accepted paradigms of carcinogenesis. We provide evidence that the extracellular DAMPs from inflammatory cells, S100A8 and S100A9, signal with colorectal cancer cells, in part via Smad4, through the RAGE receptor promoting cell proliferation and motility. Further research into S100A8/A9 signalling in the tumour microenvironment, elucidating cancer cell cytokines involved in recruiting S100A8/A9-secreting inflammatory cells, will shed light on how these proteins orchestrate crosstalk in promoting tumour development/spread.

We also showed that the intracellular DAMP, HSP27, plays an important role in maintaining migratory and proliferative activity of colorectal cancer cell, where its depletion resulted in inhibition of cell growth and motility. While high expression level of HSP27 in resected colorectal cancer specimens before 5-FU treatment correlated with poor clinical response of the cancer patients, our *in vitro* experiments showed that following HSP27 depletion, both colon and rectal cancer cell sensitivity was increased not just to 5-FU but also to irinotecan, oxaliplatin and gamma radiation. Our finding of HSP27 levels as a predictive marker of response to chemotherapy warrants validation with a large patient sample from a randomised controlled trial. This will inform the potential use of HSP27 as a biomarker in clinical practice and future drug development.

Taken as a whole, this study describes the discovery and assay development phase of S100A8, S100A9 and HSP27 as potential biomarkers in colorectal carcinogenesis. Also, our experiments delineate the roles of S100A8, S100A9

and HSP27 in carcinogenesis and yield important insights that could lead to potential therapeutic targets for colorectal cancer treatment.

CHAPTER 7:
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Carcinogenesis vol.31 no.9 pp.1541–1551, 2010
doi:10.1093/carcin/bgq137
Advance Access publication July 9, 2010

Smad4 loss is associated with fewer S100A8-positive monocytes in colorectal tumors and attenuated response to S100A8 in colorectal and pancreatic cancer cells

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S100A8 and its dimerization partner S100A9 are emerging as important chemokines in cancer. We previously reported that Smad4-negative pancreatic tumors contain fewer stromal S100A8-positive monocytes than their Smad4-positive counterparts. Here, we studied S100A8/A9-expressing cells in colorectal tumors relating their presence to clinicopathological parameters and Smad4 status. Two-dimensional gel electrophoresis (n = 12) revealed variation in the levels of S100A8 protein in colorectal cancer tumors, whereas immunohistochemical analysis (n = 313) showed variation in the numbers of stromal S100A8-positive and S100A9-positive cells. Loss of Smad4 expression was observed in 42/304 (14%) colorectal tumors and was associated with reduced numbers of S100A8-positive (P = 0.03) but not S100A9-positive stromal cells (P = 0.26). High S100A9 cell counts were associated with large tumor sizes (P = 0.0006) and poor differentiation grade (P = 0.036). However, neither S100A8 nor S100A9 cell counts predicted poor survival, except for patients with Smad4-negative tumors (P = 0.02). To address the impact of environmental S100A8/A9 chemokines on tumor cells, we examined the effects of exogenously added S100A8 and S100A9 proteins on cellular migration and proliferation of colorectal and pancreatic cancer cells. S100A8 and S100A9 enhanced migration and proliferation in Smad4-positive and Smad4-negative cancer cells. However, transient depletion of Smad4 resulted in loss of responsiveness to exogenous S100A8, but not S100A9. S100A8 and S100A9 activated Smad4 signaling as evidenced by phosphorylation of Smad2/3; blockade of the receptor for the advanced glycation end products inhibited this response. In conclusion, Smad4 loss alters the tumor's interaction with stromal myeloid cells and the tumor cells' response to the stromal chemokine, S100A8.

Introduction

The complex interaction between tumor cells and surrounding non-malignant stromal host cells is increasingly understood to be a vital regulator of cancer growth and progression (1). Bone marrow-derived myeloid cells are an important component of the tumor microenvironment. Several bone marrow-derived myeloid cells, such as macrophages, Tie-2 expressing monocytes, neutrophils and mast cells have been shown to contribute to tumor angiogenesis (2). They have also been implicated in processes of tumor invasion and metastasis [for review see Joyce *et al.* (3)]. The presence of S100A8- and S100A9-positive myeloid cells in the pancreatic cancer microenvironment was previously characterized, revealing an association between the numbers of S100A8-expressing cells and the expression in tumor cells of the tumor suppressor protein Smad4 (4). The Smad4 gene is mutated at a high frequency in pancreatic and colon cancer and to a lesser extent in a variety of other cancers (5). The microenvironment of pancreatic tumors, which lacked expression of Smad4, was found to have significantly fewer S100A8-expressing cells compared with the microenvironment of Smad4-positive tumors (4). This finding suggested a phenotypic difference in the myeloid-derived infiltrate related to the Smad4 status of these tumors.

S100A8 (calgranulin A, MRP8) and S100A9 (calgranulin B, MRP14) are low-molecular weight members of the S100 family of calcium-binding proteins, which are abundantly expressed in cells of the myeloid lineage, including monocytes and neutrophils and early-differentiation states of macrophages (6,7). They are secreted inflammatory chemoattractants that mediate further recruitment of inflammatory cells to sites of tissue damage (8) and have been implicated in a variety of chronic inflammatory conditions such as cystic fibrosis, rheumatoid arthritis, tuberculosis and transplant rejection (9,10). Recent attention has focused on the involvement of S100A8/A9 in cancer (11). These inflammatory proteins have been reported to promote tumorigenesis (12) and cause cancer metastasis by stimulating the migration of monocytes and tumor cells to metastatic sites (13,14).

In this study, stromal S100A8 and S100A9-expressing myeloid cells in colorectal tumors were thoroughly examined. The relationship between these myeloid cells and the Smad4 status of the colorectal cancers was determined as was the effect of exogenous S100A8 and S100A9 on Smad4-positive and Smad4-negative colorectal and pancreatic cancer cells.

Materials and methods

Two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, protein identification and immunodetection

Proteins were extracted from frozen colectomy specimens, separated by two-dimensional electrophoresis and S100A8 identified as described previously (4,15). Colorectal cancer tissue microarrays were obtained from the Liverpool Tissue Bank, University of Liverpool and contained duplicate or triplicate cores from 313 independent specimens of adenocarcinoma. Fourteen cores of normal colon and 10 cores each of normal kidney, liver and testis were included as control tissue. Immunohistochemistry was undertaken as described previously (16) using polyclonal goat anti-S100A8, polyclonal rabbit anti-S100A9 or monoclonal anti-Smad4 (Clone-B8; Santa Cruz Biotechnology, Heidelberg, Germany) primary antibodies. S100A8 and S100A9 antibodies detect single bands in stimulated HL60 monocytic cells (supplementary Figure 1A is available at *Carcinogenesis* Online). Co-immunofluorescence was performed on duplicate formalin-fixed colorectal tumor sections using the following primary antibodies: a monoclonal mouse anti-S100A8 and polyclonal rabbit anti-S100A9 (Santa Cruz), monoclonal mouse anti-CD68 (Dako, Ely, Cambridgeshire, UK) and monoclonal mouse anti-CD14 (Novocastra, Newcastle, UK). Microarrays were scored by a specialist histopathologist (author B.A.) and an independent evaluator. The intensity of Smad4 staining (using a 0–3 scale) was recorded and a score of ≤0.5 (mean score of at least two cores per tumor) was classified as Smad4 negative. The number of stromal cells positive for S100A8 and S100A9 cells was counted (using ×40 magnification) for each tumor core and the mean number per tumor obtained by averaging the number of positive cells across all the tumor cores scored for that patient.

Abbreviations: MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); RAGE, receptor for the advanced glycation end products; siRNA, small interfering Ribonucleic acid.

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Generation of recombinant S100A8 and S100A9 proteins

pGEX4T-1 plasmids encoding S100A8-GST and S100A9-GST (13) were kindly provided by Y.Maru, Tokyo Women's Medical University School of Medicine, Japan. Proteins were expressed in *Escherichia coli* following induction with isopropyl-b-D-thiogalactopyranoside (VWR international, Lutterworth, Leicestershire, UK) and purified with glutathione sepharose beads (GE Healthcare, Amersham, Buckinghamshire, UK). The quantity of recombinant S100A8-GST and S100A9-GST was determined by performing one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis against serial dilutions of a reference standard of bovine serum albumin (1–15 μg/lane) followed by densitometric evaluation of the Coomassie-stained gels (GS-800 scanner; Bio-Rad, Bath, UK) using QuantityOne software (Bio-Rad). All experiments using fusion proteins were performed with at least two independent batches of each protein.

Cell lines and western blotting

The rectal cell line SW837 was purchased from the European Collection of Cell Cultures in March 2008. The colonic cell line SW480 and the pancreatic adenocarcinoma cell line Panc-1 were obtained from American Type Culture Collection in 1999. Neomycin-resistant clonal derivatives of Smad4-deficient SW480 cells, stably reexpressing Smad4 (SWD20) and a negative control transfectant (SWK3), described previously (17,18) were maintained in medium containing geneticin (0.2 mg/ml;Gibco, Paisley, UK). All cell lines were last authenticated in October 2009 using short tandem repeat profiling against the international reference standard for cell line.

Western blot analysis was performed as described previously (16) using mouse anti-Smad4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Smad2, anti-phospho-Smad3, anti-phospho-Smad1/5/8 antibodies (Cell Signaling Technology, Massachusetts, MA) and mouse anti-β-actin monoclonal antibody (Sigma–Aldrich, Gillingham, Dorset, UK).

In vitro cell migration assay

Cells were plated (5×10^4 for SW837/SW480/SWD20/SWK3 and 5×10^3 for Panc-1) in medium containing 1% fetal bovine serum for Boyden Chamber assays, conducted over 18 h, as described previously (16). Recombinant proteins, S100A8-GST, S100A9-GST or GST in medium containing 1% fetal bovine serum were added to the lower transwell chambers. Migrated cells were stained and counted (16), $n = 2$ inserts per treatment, and the average number of migrated cells were calculated. Experiments were performed at least three times.

Cell proliferation assay

Cells (3×10^3/200 μl medium, supplemented with 1% fetal bovine serum) were plated in wells of 96-well plates and recombinant S100A8-GST, S100A9-GST or GST added. Proliferation was assessed at 24, 48 and 72 h using the EZ4U non-radioactive cell proliferation assay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. Absorbance readings at 450 nm were taken at 4 h following incubation with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagents. Experiments were performed at least three times and five wells utilized for each treatment.

Cellular phospho-Smad expression and blocking of receptor of advanced glycation end products with specific blocking antibody

Cells were cultured for 48 h in serum-free medium supplemented with bovine serum albumin (10 μg/ml; Sigma–Aldrich) and human insulin (5 μg/ml; Sigma–Aldrich) and then treated with S100A8-GST, S100A9-GST, GST or transforming growth factor-β (10 ng/ml; PeproTech EC Ltd, London, UK) for 1 h, followed by cell collection for western blotting as described above. For receptor of advanced glycation end products (RAGE) blocking, cells were treated with RAGE-blocking antibody (40 or 80 μg/ml; R&D Systems, Abingdon, UK) for 1 h before the addition of recombinant proteins.

Transient Smad4 knockdown

Small interfering Ribonucleic acid (siRNA) experiments were performed as described previously (16). Smad4-targeting siRNAs, GUGUGCAGUUG-GAAUGUAA (Smad4 siRNA1) and GUACAGAGUUACUACUUAG (Smad4 siRNA2) were purchased from Dharmacon (Chicago, IL). Two non-targeting control siRNAs were used, control 1 (siControl non-targeting siRNA 1 from Dharmacon) and control 2 (GGACGCAUCCUUCUUAA, a gift from M.Boyd, University of Liverpool, UK). Smad4 levels diminished between 48 and 72 h post-transfection with Smad4-targeting siRNAs and remained low out to 120 h (data not shown).

Cellular immunofluorescence

Cells were fixed with 4% formaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min. Phospho-Smad2/3 was detected with anti-phospho-Smad2/3 antibody (Cell Signaling Technology) and visualized with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories West Grove, PA). Actin filaments were labeled with phalloidin (Invitrogen, Renfrew, UK).

Statistical analysis

Comparisons between two groups were made using nonparametric continuity corrected chi-square test or chi-squared test, when more than two groups were analyzed. Continuous variables were compared using the Mann–Whitney *U*-test or the Wilcoxon signed-rank test. To evaluate the effect of stromal S100A8 or S100A9 cells on patient survival, life tables were constructed and Kaplan–Meier curves plotted. Overall survival was measured from date of initial surgery to date of death, counting death from any cause as the end point or the last date of information as the end point if no event was documented. To analyze data from 'motility' or 'proliferation' experiments, continuous variables were compared using the Student's *t*-test and were expressed as mean. All analyses were performed using Statview Version 5.01 (SAS Institute Cary, North Carolina). A *P*-value of <0.05 was considered significant.

Results

Variable S100A8 and S100A9 levels in colorectal cancer microenvironments

Two-dimensional gels displaying proteins extracted from 12 individual undissected colorectal tumors revealed variation (Figure 1A–C) in the intensity of a spot (arrowed in Figure 1A and B) that was suspected, on the basis of its gel location, to contain S100A8. Protein recovered from this spot was trypsin digested and analyzed by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (Figure 1D). This resulted in the detection of five peptides, providing sequence coverage of 37.6% and confirmed the identification of S100A8 (National Centre for Biotechnology Information accession no. CAG28602). To establish the cellular basis for the variability of S100A8, immunohistochemistry of formalin-fixed colorectal adenocarcinomas was undertaken, for the detection of S100A8 and its dimerization partner S100A9. This revealed the expression of both proteins in cells scattered throughout the tumor stroma [Figure 2A(i–iv)]. While S100A9 expression was not detected in cancer cells, a very low intensity of cytoplasmic S100A8 staining was observed in tumor cells of virtually all cases and was not subjected to analysis. Co-immunofluorescence indicated extensive colocalization of S100A8 and S100A9 in stromal cells [Figure 2B(i)]. Furthermore, co localization of S100A8 or S100A9 was observed in some cells with the monocyte marker CD14 [Figure 2B(ii and iii), respectively] but not the macrophage marker CD68 [Figure 2B (iv and v), respectively).

The number of S100A8-positive or S100A9-positive cells in each of 313 patient tumors (mean of at least two Tissue Microarray cores per tumor case) was determined. Numbers ranged from 0 (4.5% of cases) to 288 and from 0 (0.65% of cases) to 882 for S100A8 and S100A9, respectively. The median number of stromal S100A8-expressing cells was 23 [Inter-quartile range (IQR) 6–70], whereas the median number of stromal S100A9-expressing cells was 65 (IQR 27–126) with 284/308 (92%) tumors showing more S100A9 than S100A8-expressing cells in the stroma. Although tumors generally contained fewer S100A8-positive than S100A9-positive cells ($P < 0.0001$, Wilcoxon signed-rank test), there was a strong positive relationship between S100A8 and S100A9 counts ($n = 302$ independent tumor cases; $R^2 = 0.76$, $P < 0.0001$). This is entirely consistent with our observation that S100A8 colocalized with S100A9. The median S100A9:S100A8 ratio was 2.2 (IQR 1.3–4.4). To examine whether a relationship existed between the numbers of stromal S100A8-positive or S100A9-positive cells and the Smad4 tumor status, the expression of Smad4 protein was determined by immunohistochemistry [Figure 2A (v and vi)]. Forty-two of 304 patients (14%) were categorized as Smad4 negative based on mean cytoplasmic intensity scores of ≤ 0.5. The remaining 262 of 304 patients (86%) were categorized as Smad4 positive. Loss of Smad4 expression in primary colorectal tumor cells was associated with a significantly lower median count of S100A8-positive stromal cells (14, IQR 5–37) compared with a median S100A8-positive cell count of 25 (IQR 6–76) in the Smad4-positive group [Figure 2C (i)], $P = 0.03$, Mann–Whitney *U*-test). A similar relationship was not observed between

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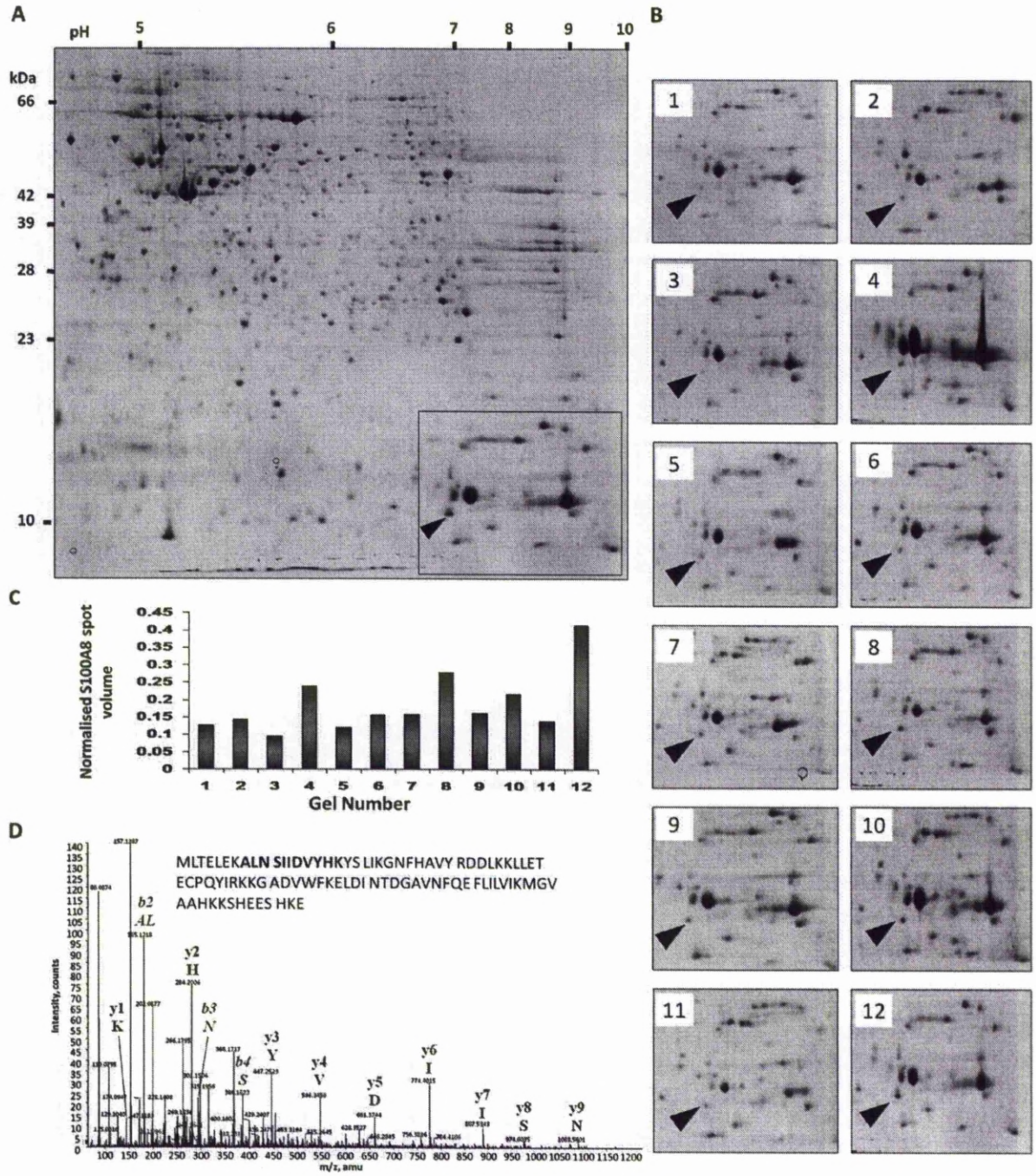

Fig. 1. (**A**) Colloidal Coomassie Blue-stained two-dimensional gel image of a colorectal cancer lysate, with the S100A8-containing spot arrowed. (**B**) Insets from 12 independent colorectal tumor gels showing different intensities in the S100A8 protein spot. (**C**) Normalized S100A8 levels (S100A8 spot intensity/total spots intensity). (**D**) Mass Spectrometry/Mass Spectrometry spectrum of the peptide ALNSIIDVYHK from S100A8.

tumor cell Smad4 expression and S100A9-positive stromal monocytes [Figure 2C (ii)], $P = 0.26$).

Relationship between S100A8/A9-positive stromal cells and clinicopathological characteristics

To examine for associations between stromal S100A8-positive cells and patient clinicopathological parameters, patients were categorized as having mean S100A8-positive cell counts that were low ($\leq$ median of 23) or high (>23). Similarly, for S100A9, patients were categorized as having low ($\leq$ median of 65) or high (>65) cell counts. The degree of stromal S100A8/A9-positive infiltration showed no correlation with the parameters of age at surgery, gender, site of tumor, depth of tumor invasion or nodal metastases (Table I). However, high cell S100A8 and S100A9 counts were

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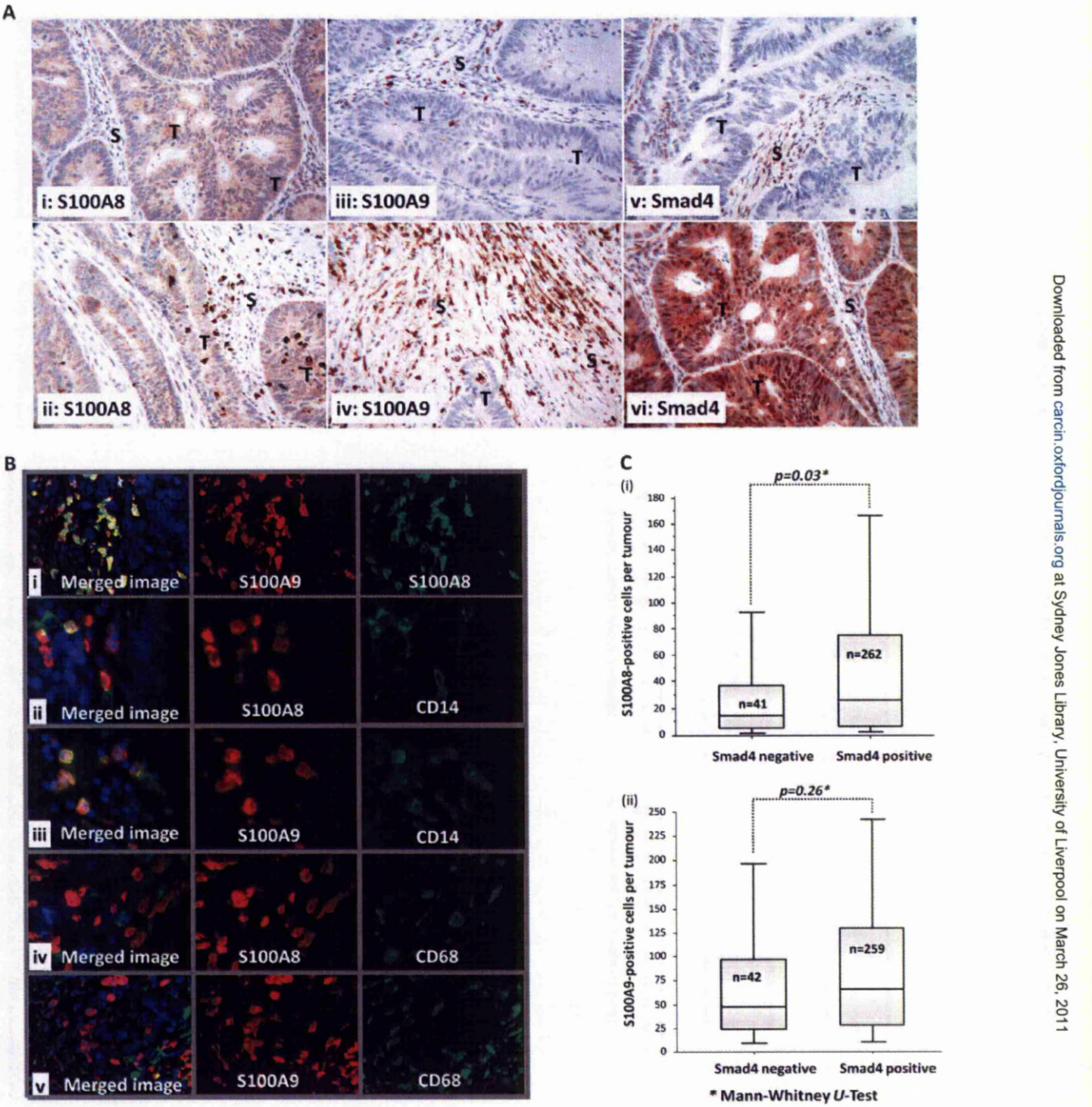

Fig. 2. Colorectal cancer tissue illustrating a low [**A**(i and iii)], and a high [A(ii and iv)] infiltrate of S100A8- and S100A9-positive cells, respectively; the absence and presence, respectively, of Smad4 staining [A(v and vi)]; colocalization of S100A9 and S100A8 [B(i)], S100A8 or S100A9 and CD14 [**B**(ii and iii)]; lack of colocalization of S100A8 or S100A9 and CD68 [B (iv and v)]. The mean numbers of S100A8-positive [C(i)] or S100A9-positive cells [**C**(ii)] per tumor were plotted for Smad4-negative and Smad4-positive tumors.

associated with larger tumor size ($P = 0.01$ and 0.0006, respectively). These proteins are co-expressed to a high degree. Nevertheless, we wished to determine whether either protein was independently associated with tumor size. We therefore performed logistic regression analyses. Both high S100A8 and high S100A9 cell counts were associated with large tumor size on univariate analysis ($P = 0.01$ and 0.0007, respectively; supplementary Table 1 is available at *Carcinogenesis* Online). However, on multivariate analysis, only high S100A9 cell counts remained independently associated with large tumor sizes ($P = 0.02$). High S100A9 cell counts were also associated with poor differentiation grade ($P = 0.036$).

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Table I. Clinicopathological characteristics and correlation with S100A8 and S100A9 expression

	All cases, $n = 313$ (%)	S100A8-positive cells, $n = 309$		P value[a]	S100A9-positive cells, $n = 306$		P value[a]
		Low ≤ 23 cells (%), $n = 156$	High > 23 cells (%), $n = 153$		Low ≤ 65 cells (%), $n = 159$	High > 65 cells (%), $n = 147$	
Age 70 (IQR 62–76 years)							
Young (<median)	155 (49)	75 (48)	80 (52)	0.459	75 (47)	78 (53)	0.303
Old (>median)	158 (51)	81 (52)	73 (48)		84 (53)	69 (47)	
Gender							
Male	188 (60)	93 (60)	92 (60)	0.926	97 (61)	85 (58)	0.570
Female	125 (40)	63 (40)	61 (40)		62 (39)	62 (42)	
Site of tumor							
Colon	188 (60)	89 (57)	96 (63)	0.307	94 (59)	90 (61)	0.707
Rectum	125 (40)	67 (43)	57 (47)		65 (41)	57 (39)	
Size 50 (IQR 38–60 mm)							
Small-medium (<60 mm)	214 (69)	116 (74)	94 (61)	**0.014**	123 (77)	87 (59)	**0.0006**
Large (≥60 mm)	99 (31)	40 (26)	59 (39)		36 (23)	60 (41)	
Differentiation grade							
Well	5 (2)	2 (1)	2 (1)	0.819	2 (1)	3 (2)	**0.036**
Moderate	283 (90)	140 (90)	140 (92)		150 (94)	127 (86)	
Poor	21 (7)	12 (8)	9 (6)		5 (4)	15 (11)	
Uncategorized/DNA	4 (1)	2 (1)	2 (1)		2 (1)	2 (1)	
Excision margin							
Clear	270 (86)	130 (83)	138 (90)	0.055	134 (84)	130 (88)	0.199
Involved	39 (13)	25 (16)	12 (8)		24 (15)	14 (10)	
Uncategorized/DNA	4 (1)	1 (1)	3 (2)		1 (1)	3 (2)	
T-stage							
T1	13 (4)	8 (5)	5 (3)	0.766	8 (5)	5 (3)	0.356
T2	47 (15)	22 (14)	25 (16)		21 (13)	24 (16)	
T3	206 (66)	104 (67)	101 (66)		110 (69)	92 (63)	
T4	44 (14)	19 (12)	22 (15)		18 (12)	25 (17)	
Uncategorized/DNA	3 (1)	3 (2)	0		2 (1)	1 (1)	
N-stage							
N0	177 (56)	82 (53)	93 (61)	0.446	90 (57)	82 (56)	0.332
N1	71 (23)	38 (24)	32 (21)		40 (25)	30 (20)	
N2	62 (20)	33 (21)	28 (18)		27 (17)	34 (23)	
Uncategorized/DNA	3 (1)	3 (2)	0		2 (1)	1 (1)	
AJCC/UICC stage groupings							
I	39 (13)	22 (14)	17 (11)	0.319	21 (13)	17 (12)	0.860
II	136 (43)	60 (38)	74 (48)		68 (43)	65 (44)	
III	132 (42)	70 (45)	60 (39)		66 (41)	63 (43)	
Uncategorized/DNA	6 (2)	4 (3)	2 (2)		4 (3)	2 (1)	
Chemoradiotherapy							
Neoadjuvant only	35 (11)	21 (13)	14 (9)	0.630	19 (12)	15 (10)	0.724
Adjuvant only	77 (25)	37 (24)	40 (26)		37 (23)	40 (27)	
Neoadjuvant and adjuvant	12 (4)	6 (4)	5 (3)		6 (4)	4 (3)	
No chemoradiotherapy	188 (60)	92 (59)	93 (61)		97 (61)	87 (59)	
DNA	1 (0.3)	0 (0)	1 (1)		0 (0)	1 (1)	

AJCC/UICC, American Joint Committee on Cancer/International Union Against Cancer; DNA, data not available.
[a]Chi-squared test.

Neither S100A8-positive nor S100A9-positive stromal cell counts were associated with patient survival [Figure 3A (i and ii)]. However, for the smaller cohort of patients exhibiting loss of Smad4 expression, high S100A8 or S100A9 cell counts predicted poor 3 year survival [Figure 3B (i and ii)]. The number of Smad4-negative tumor patients in the study was too small to determine whether either of the proteins was independently associated with poor outcome. No survival difference was observed for either high S100A8 or S100A9 counts at 60 months in this Smad4-negative cohort. For patients with Smad4-positive tumors, neither S100A8-positive nor S100A9-positive counts predicted overall short- or long-term survival [Figure 3C (i and ii)].

The effects of Smad4 status on S100A8- and S100A9-induced migration activity

S100A8 and S100A9 are secreted chemokines, which could affect tumor cells in their vicinity. We next investigated their chemoattractive functions on tumor cells *in vitro*. The addition of purified S100A8-GST and S100A9-GST fusion proteins (see supplementary Figure 1B, available at *Carcinogenesis* Online) at concentrations of either 0.4 or 2 μg/ml significantly increased the migration activity of the rectal cancer cell line SW837, the colon cancer cell line SW480 and the pancreatic cancer cell line Panc-1 (Figure 4A) ($P < 0.001$) compared with the addition of purified GST control protein. The addition of both S100A8-GST and S100A9-GST proteins simultaneously did not significantly increase the response over proteins added individually (see supplementary Figure S2, available at *Carcinogenesis* Online) and may be due to failure of the recombinant proteins to interact correctly.

Both Panc-1 and SW837 cells express Smad4 (see inset Figure 4A). To determine whether the Smad4 status of cells influenced their response to S100A8 or S100A9, we depleted Smad4 in these cell lines and examined their migration activity in response to S100A8 and S100A9. An ~50% decrease in basal migration in Panc-1 cells compared with control siRNA-treated cells was observed (Figure 4B), consistent with previously published data (19). Smad4 knockdown totally suppressed migration responses toward S100A8. In contrast, S100A9-treated cells retained an ~2-fold increase in migration after

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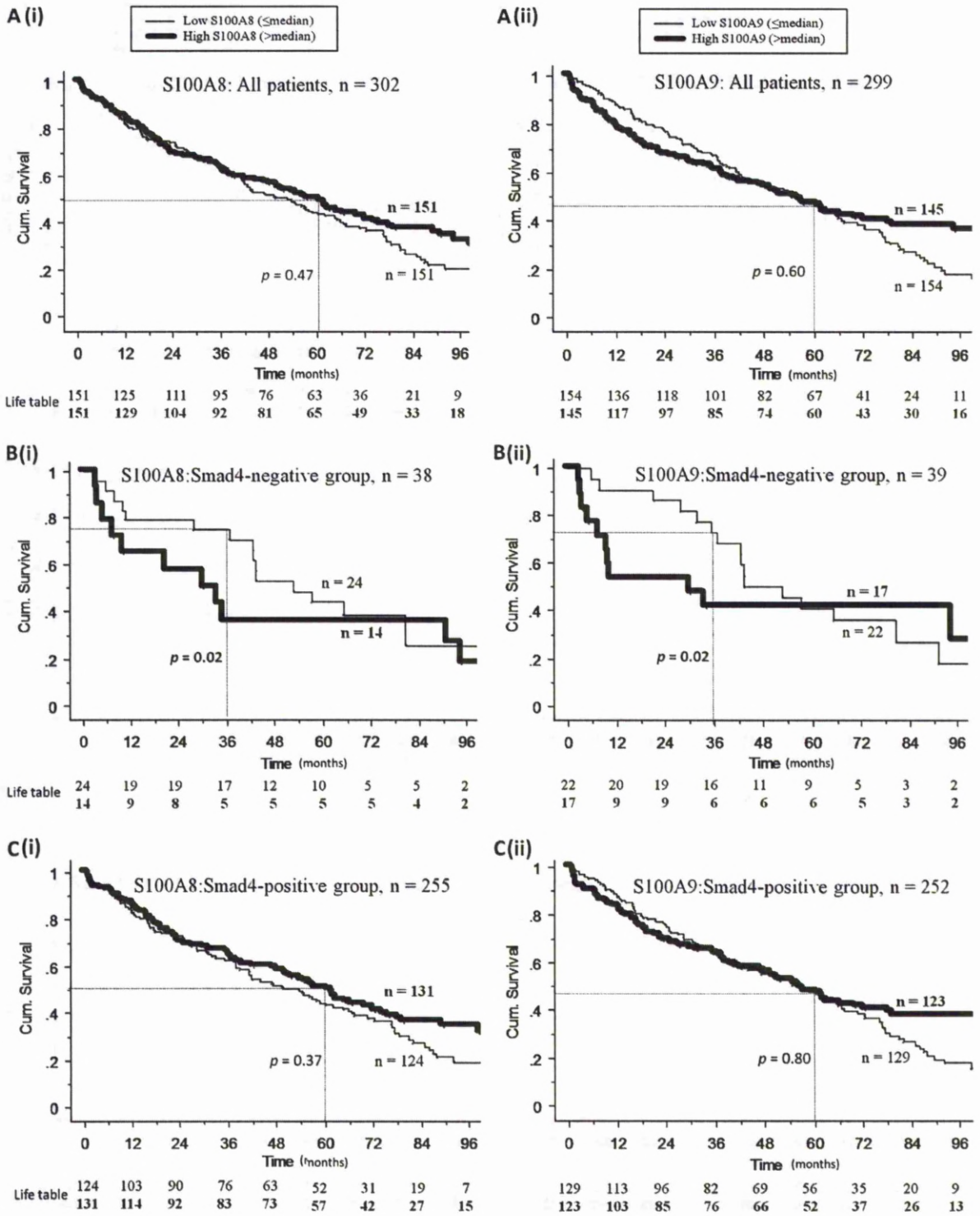

Fig. 3. Kaplan–Meier graphical analysis showing (**A**) no survival difference based on S100A8 [A(i)] or S100A9 [A(ii)] cell counts in all patients, (**B**) a survival difference in patients with Smad4-negative tumors based on S100A8 [B(i)] or S100A9 [B(ii)] cells counts and (**C**) no survival difference in patients with Smad4-positive tumors based on S100A8 [C(i)] or S100A9 [C(ii)] cell counts.

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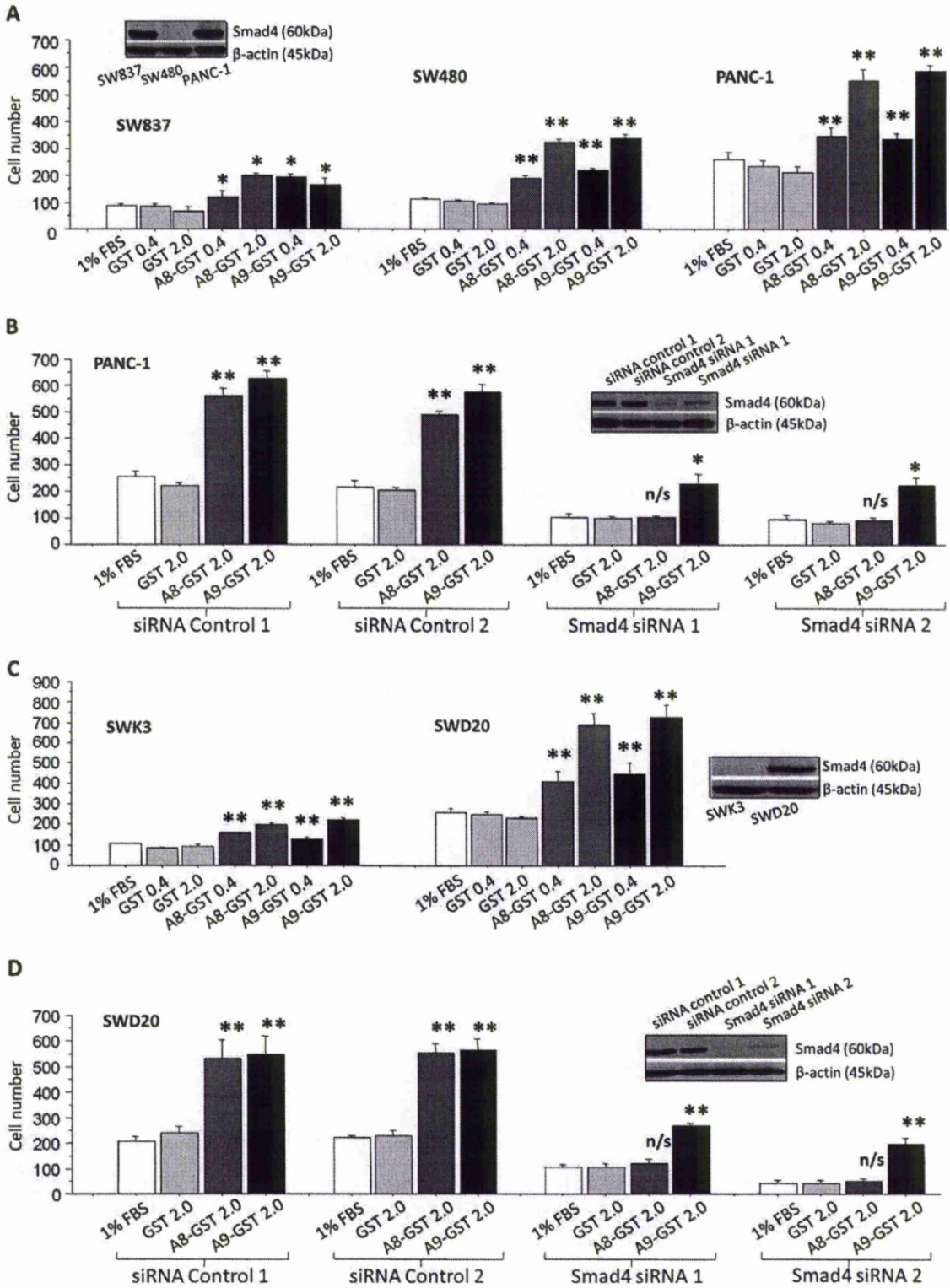

Fig. 4. Migration activities of indicated cell lines treated with S100A8-GST (A8-GST), S100A9-GST (A9-GST), GST at concentrations of 0.4 or 2.0 μg/ml, in medium containing 1% fetal bovine serum (FBS) (**A**) or Smad4-depleted Panc-1 cells (**B**) or clonal derivative of SW480 cells that stably reexpresses Smad4 (SWD20) and its Smad4-negative control (SWK3) (**C**) or Smad4-depleted SWD20 (**D**). $^{**}P < 0.001$, $^{*}P < 0.05$. The Smad4 status of cells is shown as insets on western blots.

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Smad4 knockdown. Smad4 knockdown caused an almost total loss of basal and chemokine-induced migration of the less motile SW837 cells (supplementary Figure 3 is available at *Carcinogenesis* Online).

SW480 cells do not express Smad4 (see inset Figure 4A). Neomycin-resistant clonal derivatives of SW480 cells, stably reexpressing Smad4 (SWD20) and negative control transfectants (SWK3) (17,18) were used to determine whether restoration of Smad4 would alter responsiveness to S100A8 or S100A9. We found that both the Smad4-negative and Smad4-positive subclones had enhanced migration following incubation with S100A8-GST or S100A9-GST (Figure 4C). However, transient siRNA-mediated depletion of Smad4 from SWD20 cells resulted in a reduction in motility and a loss of responsiveness to S100A8-GST, but not S100A9-GST (Figure 4D). This was similar to the result obtained with Panc-1 cells (Figure 4B).

The effects of Smad4 status on S100A8- and S100A9-induced proliferation

Similarly, we sought to examine the effects of S100A8 and S100A9 on tumor cell proliferation and explore whether Smad4 status was important in this context. Incubation of SW837 and SW480 cells with 2 μg/ml of S100A8-GST or S100A9-GST resulted in modest, though statistically significant increases in MTS readings (Figure 5A). No increase was observed using 0.4 μg/ml recombinant proteins (data not shown) and Panc1 cells did not show an increased MTS response at all (supplementary Figure 4A, available at *Carcinogenesis* Online). In the case of SW837 cells, the simultaneous addition of S100A8-GST and S100A9-GST proteins at 2 μg/ml significantly improved the response over proteins added individually (see supplementary Figure 4B, available at *Carcinogenesis* Online), although this effect was not observed with SW480 or Panc-1 cells (supplementary Figure 4C and D is available at *Carcinogenesis* Online).

The Smad4-negative SW480 subclone, SWK3 showed enhanced proliferation in response to 2 μg/ml S100A9-GST (Figure 5B), whereas the Smad4 reexpressing subclone, SWD20, responded to both S100A8-GST and S100A9-GST, although the response to S100A9-GST was greater (Figure 5B). Following depletion of Smad4 from SWD20 cells, responsiveness to S100A8-GST was lost (Figure 5C). S100A9-GST continued to induce proliferation, although the extent of proliferation reached was not as great as that observed in control siRNA-treated cells (Figure 5C). Depletion of Smad4 from Panc-1 cells gave a similar result (supplementary Figure 5 is available at *Carcinogenesis* Online) although the increase in MTS readings in response to S100A9 was very small.

The effects of exogenous S100A8 and S100A9 on Smad4 signaling and RAGE

Our results indicated that the effects of exogenous S100A8 and S100A9 were influenced to an extent by the presence of Smad4 in tumor cells. To examine whether these proteins could signal via Smad4, cells were incubated for 1 h with 2 μg/ml of S100A8-GST or S100A9-GST and the activation of members of the Smad pathway examined. Both recombinant S100 proteins alone, but not GST, induced increased levels of phospho-Smad2, phospho-Smad3, but not phospho-Smad1/5/8 in Panc-1 cells [Figure 5D (i)]. Furthermore, phospho-Smad2/3 was shown to accumulate in the nucleus following incubation with S100A8-GST or S100A9-GST, but not GST (supplementary Figure 6 is available at *Carcinogenesis* Online). Similar results were obtained with SWD20 cells (data not shown). S100A8/A9 heterodimers are known to stimulate cells through binding to the cellular receptor RAGE (20,21), although it is unclear whether the individual proteins are ligands for this receptor. RAGE expression was observed in the cell lines used in this study [Figure 5D (ii)]. To determine whether S100A8 and S100A9 were activating the Smad pathway through RAGE, we used a RAGE-blocking antibody at two concentrations, 40 and 80 μg/ml. When Panc-1 cells were pretreated with the RAGE-blocking antibody, a dose-dependent reduction of phopho-Smad2 and phospho-Smad3 levels was observed in response to S100A8-GST or S100A9-GST stimulation [Figure 5D (iii)].

Discussion

The inflammatory chemoattractants, S100A8 and S100A9, apart from serving as markers of phagocytes (intracellular S100A8/A9) or as biomarkers of inflammatory disease conditions (secreted extracellular S100A8/A9), are now recognized to play important roles themselves in the pathogenesis of inflammatory disorders and more recently in cancer (11). The presence of S100A8- and S100A9-positive cells has been reported previously in smaller studies of colorectal cancer (12,22). Here, we provide a comprehensive analysis, in which we quantified the numbers of S100A8/A9 cells infiltrating the environment of colorectal tumors. We also noted expression of S100A8/A9 proteins in neutrophils localized in the tumor vasculature, although this cell type was not studied further. S100A8 and S100A9 proteins were largely co-expressed in the same cells, and the majority of tumors (92%) contained more S100A9-positive cells than S100A8-positive cells, which may reflect the differentiation state of the myeloid cells expressing these proteins (23). There was overlap in the expression of S100A8 and S100A9 and the monocyte/macrophage marker CD14, although the S100A8/A9-positive cell population did not express the mature macrophage marker CD68, indicating that the S100A8/A9 population may be at an early stage of monocyte/macrophage maturation.

Relatively little is known about tumor-associated monocytes in colorectal cancer, although $CD68^{+}$ tumor-associated macrophages have been analyzed in detail. High infiltrates of $CD68^{+}$ tumor-associated macrophages in colorectal tumors were shown to correlate with absence of vascular and lymph node invasion (24) and improved survival (25,26). No evidence was found for an association between the numbers of S100A8/A9-positive cells and either depth of tumor invasion or nodal metastases. High S100A9 infiltrate, however, was associated with poor differentiation grade and correlated strongly with larger tumor size. In contrast to the reported links between high $CD68^{+}$ infiltrate and improved outcome, when the entire cohort of ~300 patients was examined as a whole, no association was found between S100A8/A9 levels and survival. Nonetheless, the short-term survival of patients with Smad4-negative tumors and high A8/A9 infiltrate was significantly poorer than Smad4-negative patients with low A8/A9 infiltrate. This suggests that Smad4-negative tumors have a shorter time to recurrence or metastases in the presence of high stromal S100A8- or S100A9-expressing monocytes. Mutations or inactivation of Smad4 in colorectal carcinoma coincide with progression to metastatic disease; the highest percentages of inactivation (>30%) are observed in patients with distant organ metastasis (27). In our study, only non-metastatic tumors with stages I–III disease were included, which accounts for the lower frequency of Smad4 loss (14%) in our cohort.

One of the main aims of our study was to determine if the extent of the S100A8/A9-positive infiltrate was influenced by the Smad4 status of the colorectal tumors under examination. The motivation to examine this stemmed from the previous observation that Smad4-negative pancreatic tumors contained fewer S100A8-positive stromal cells than Smad4-negative pancreatic tumors (4). Furthermore, using a mouse model of colon cancer, Kitamura *et al.* (28) reported that Smad4-negative tumors recruited a specific type of myeloid cell, which promoted invasion through cross talk with tumor cells. We observed a distinctive reduction in the numbers of S100A8-positive, but not S100A9-positive stromal cells in Smad4-negative colorectal tumors, resulting in a change in the relative levels of S100A8 to S100A9. The S100A8/A9 myeloid cell population that we have studied does not appear to be identical to that observed in the mouse study of Kitamura *et al.* (28) because the mice cells lacked expression of CD14. However, our observation that the loss of Smad4 is accompanied by a change to the phenotype of the myeloid infiltrate is consistent with that of Kitamura *et al.* and provides important evidence in human colorectal cancer that the phenotype of the myeloid infiltrate is influenced by the Smad4 status of the tumor.

Hiratsuka *et al.* (13) showed that S100A8/A9 were powerful chemoattractants whose tumor-induced presence in the lungs of tumor-bearing mice could stimulate the migration of Lewis Lung carcinoma

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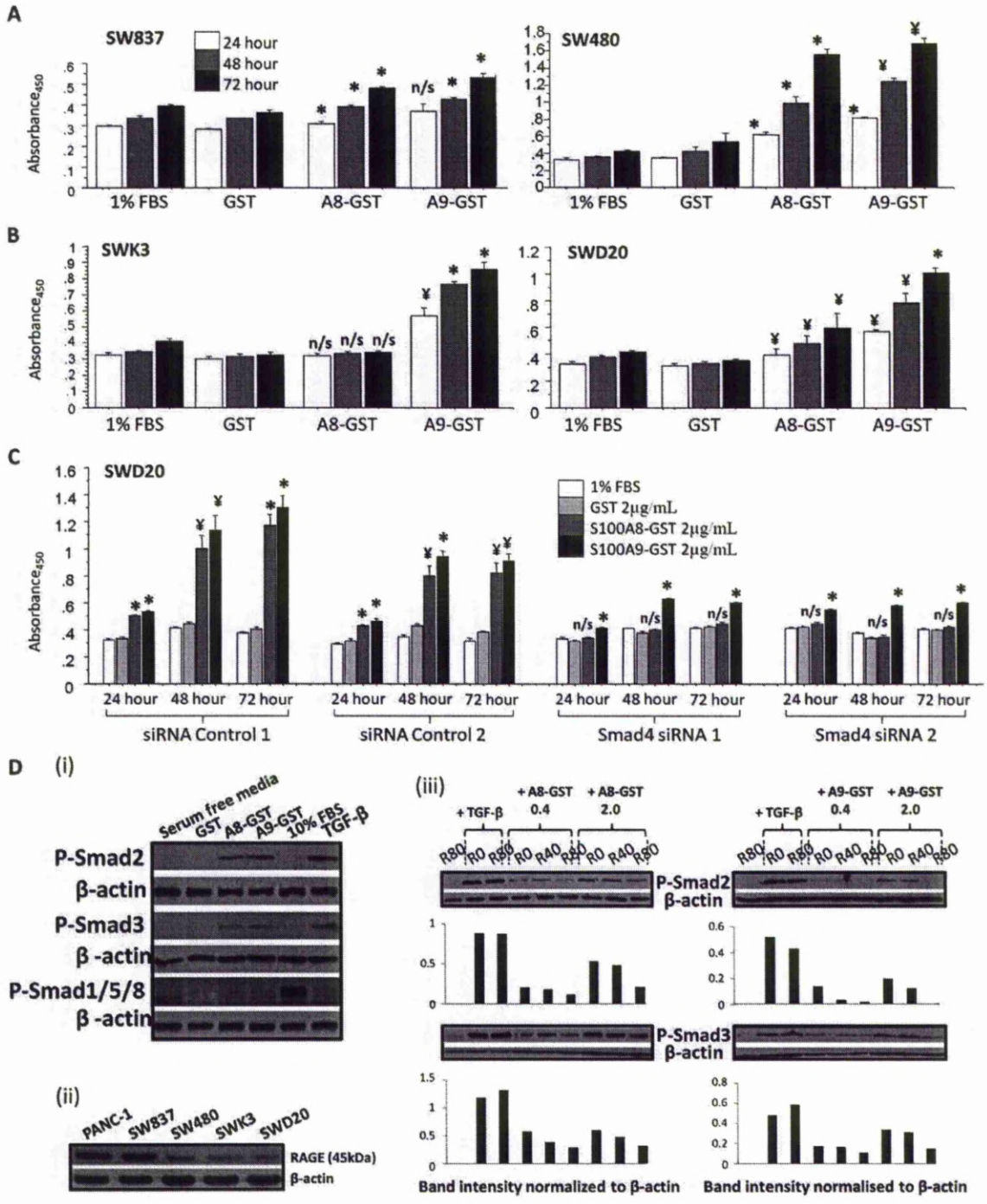

Fig. 5. MTS readings of indicated cells in response to S100A8-GST (A8-GST), S100A9-GST (A9-GST) or GST at a concentration of 2.0 μg/ml (**A**) or SW480 derivatives (**B**) or Smad4-depleted SWD20 (**C**). $^{*}P < 0.01$, $¥P < 0.05$. Western detection of phospho-Smad2, phospho-Smad3 and phospho-Smad1/5/8 levels [**D**(i)] following treatment of Panc-1 cells with 2 μg/ml of S100A8-GST (A8-GST), S100A9-GST (A9-GST) or GST. Transforming growth factor (TGF-β) (10 ng/ml) and 10% fetal bovine serum (FBS) were the positive control for phospho-Smad2/3 and phospho-Smad1/5/8 activation, respectively. Western detection of RAGE expression [**D**(ii)]. Western detection and densitometric representation of the levels of phospho-Smad2 and phospho-Smad3, which were reduced in Panc-1 cells pretreated with RAGE-blocking antibody (R) (40 and 80 μg/ml) before the addition of 0.4 or 2 μg/ml of S100A8-GST or S100A9-GST [D(iii)].

cells and B16 melanoma cells to that organ, where they formed secondary tumors. We sought to determine whether the response of tumor cells to S100A8/A9 was potentially influenced by their Smad4 status. Both S100A8 and S100A9 were highly chemotactic for rectal, colon and pancreatic cancer cell lines, regardless of whether the cells expressed Smad4 or not. In Smad4-expressing cells, such as Panc-1 cells

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or the SWD20 cells, where Smad4 expression has been stably restored, transient depletion of Smad4 expression was accompanied by a loss of responsiveness to S100A8-induced migration activity but not that of S100A9, suggesting that S100A8 elicits its response through a Smad4-dependent pathway. Transient Smad4 depletion, such as we undertook in our siRNA experiments, may not allow sufficient time for cells to adapt and to allow S100A8 to signal in a Smad4-independent manner. Our observation of increased levels of phosphor-Smad2 and phospho-Smad3 in response to cell treatment with S100A8 and S100A9 provide supporting evidence that these proteins can activate the Smad4 signaling pathway.

S100A8/A9-induced proliferation of the cancer cell lines studied was also observed, although at concentrations higher that those required to induce migration activity. S100A8/A9 may contribute to apoptosis (11), however, the apoptotic role of these proteins was not investigated in this study. Ghavami *et al.* (21) reported that S100A8 and S100A9 proteins promoted growth of human breast cancer and neuroblastoma cells and confirmed that these proteins activate the multiligand receptor, RAGE, triggering the mitogen-activated protein kinase signaling pathway. The mitogenic effects of S100A8/A9, coupled with our observation that high levels of A8/A9 infiltrate were associated with larger tumors suggest that the proteins may contribute to the growth of these tumors. Interestingly, while the Smad4-restored SWD20 cells showed increased proliferation in response to S100A8-GST and S100A9-GST, their Smad4-negative clonal counterpart, SWK3 cells showed increased proliferation in response to S100A9-GST only, suggesting that Smad4 is important in regulating the proliferative response to exogenous S100A8. Transient Smad4 knockdown in SWD20 and Panc-1 was accompanied by a loss of response to S100A8-GST. Thus, although the measured proliferation effects of S100A8/A9 were not as marked as their chemotactic effects, the dependence on Smad4 for S100A8 signaling was a recurrent theme. Our RAGE-blocking experiments provide evidence that S100A8 and S100A9 activation of the Smad4 pathway occurs at least in part through RAGE. The ability of Advanced Glycation Endproducts to activate transforming growth factor-β signaling via RAGE and mitogen-activated protein kinases has been established by Li *et al.* (29).

Monocytes, once recruited from blood initially express both S100A8 and S100A9 and as they mature, they lose S100A8 expression, leaving only S100A9, which is also subsequently lost as the cell matures further (30). We postulate that myeloid cells (such as monocytes) expressing both S100A8 and S100A9 are recruited to the tumor microenvironment of Smad4-positive tumors, where they secrete S100A8/A9 that may promote further recruitment of inflammatory cells as well as cancer cell growth and invasion. In the case of Smad4-negative tumors, our data suggest that the myeloid cells recruited express S100A9, but exhibit lower S100A8 expression. Alternatively, the myeloid cells recruited are similar in the case of Smad4-positive and Smad4-negative tumors, however, the rate of differentiation, once recruited to the environment of Smad4-negative tumors is different, such that S100A8 expression becomes very transient, leaving relatively greater numbers of cells expressing only S100A9. Either way, our experiments indicate that the cross talk between Smad4-negative cancer cells and myeloid cells occurs in an environment that involves S100A9 to a greater extent than S100A8 and where the cancer cells respond better to S100A9 than S100A8. Further research into S100A8/A9 signaling in the tumor microenvironment will shed light on how these proteins may influence the processes of tumor development/spread and will provide opportunities for targeted intervention.

Supplementary material

Supplementary Figures 1–6 and Table 1 can be found at http://carcin.oxfordjournals.org/

Funding

National Institute for Health Research Liverpool Pancreatic Biomedical Research Unit; Liverpool Experimental Cancer Medicine Centre; Royal College of Surgeons of England, UK; Pancreatic Cancer Research Fund, UK; European Framework 6 Integrated project grant (LSHB-CT-2006-018771).

Acknowledgements

Conflict of Interest Statement: None declared.

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Received February 6, 2010; revised May 29, 2010; accepted June 29, 2010

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Low molecular weight heat shock protein HSP27 is a prognostic indicator in rectal cancer but not colon cancer

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Accepted 4 June 2010

ABSTRACT

Objective There are currently no biomarkers in routine clinical use for determining prognosis in rectal cancer. In a preliminary proteomic study, variation in the levels of heat shock protein 27 (HSP27) in colorectal cancer samples was observed. The expression of HSP27 in a cohort of 404 patients with colorectal cancer with a predominantly poor prognosis was characterised and an investigation was undertaken of whether the differences were related to clinical outcome. HSP27 levels in diagnostic rectal biopsies were compared with matched surgical samples to determine whether changes in expression occurred in the time between biopsy and surgery and to investigate whether preoperative radiotherapy affected expression. Finally, the relationship between HSP27 expression and outcome was examined in an independent cohort of 315 patients with a predominantly good prognosis.

Methods HSP27 levels were determined using combined two-dimensional gel electrophoresis and tandem mass spectrometry (12 cases) and by immunohistochemistry using tissue microarrays of colorectal cancers sampled at surgery and 80 diagnostic rectal biopsies.

Results HSP27 overexpression was strongly associated with poor cancer-specific survival in rectal cancer (n=205, p=0.0063) but not colon cancer (n=199, p=0.7385) in the cohort with a poor prognosis. Multivariate Cox regression confirmed nodal metastases (p=0.0001) and HSP27 expression (p=0.0233) as independent markers of survival in rectal cancer. HSP27 levels remained unchanged in the majority of cases (65/80, 81%) between diagnostic biopsies and matched surgical samples, regardless of whether patients had undergone preoperative radiotherapy. In the cohort with a good prognosis the association between HSP27 and survival was not observed in patients with either rectal (n=115; p=0.308) or colon cancer (n=200; p=0.713).

Conclusion In a large cohort of patients with a poor prognosis, HSP27 is an independent marker of poor outcome in rectal cancer; its expression is not altered by neoadjuvant radiotherapy. This finding requires validation in an independent similar cohort of patients with rectal cancer. HSP27 levels merit evaluation as a stratification factor for treatment of rectal cancer.

Significance of this study

What is already known about this subject?

- Prognostic markers that will inform the treatment of patients with rectal cancer are badly needed.
- HSP27 is a well-characterised anti-apoptotic protein.
- The prognostic value of HSP27 has been demonstrated in some tumours such as prostate cancer.

What are the new findings?

- Analysis of a cohort of 404 patients with colorectal cancer showed that HSP27 overexpression was strongly associated with poor cancer-specific survival in rectal cancer (n=205, p=0.0063) but not colon cancer (n=199, p=0.7385).
- In a smaller independent cohort of patients with colorectal cancer (n=315) characterised by earlier stage disease and significantly better overall survival, a relationship between HSP27 expression and outcome was not observed in either rectal cancer (n=115; p=0.308) or colon cancer (n=200; p=0.713).
- HSP27 levels remain largely unchanged between diagnostic biopsies and matched surgical samples and do not appear to be altered by administration of neoadjuvant therapy.

How might it impact on clinical practice in the foreseeable future?

- Our finding merits validation in a large cohort of patients with rectal cancer in whom treatment has been standardised.
- Patients with stage II rectal cancer and high HSP27 should be considered for adjuvant therapy.

INTRODUCTION

Globally, around one million people per year are diagnosed with colorectal cancer.[1] Two-thirds of cases have cancer of the colon and the remaining one-third have cancer of the rectum. The primary prognostic variables for patients with this disease remain depth of invasion and extent of nodal involvement.[2] Nonetheless, 20% of node negative

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patients have disease recurrence, indicating the need for additional prognostic biomarkers that can complement clinical staging. For locally advanced rectal cancer, preoperative therapy is standard treatment and can result in significant downstaging and downsizing of tumours.[3 4] Prognostic biomarkers measurable in the preoperative biopsy are wanted to facilitate the selection of patients for treatment. We describe the first comprehensive analysis of heat shock protein HSP27 expression in colorectal cancer and its relationship to prognosis.

Heat shock proteins are ubiquitous molecular chaperones that fold new proteins correctly, enabling them to attain their functional conformation. They also prevent existing proteins from aggregating under conditions of stress.[5 6] A variety of stimuli—including heat, oxidative stress or exposure to chemotherapeutic compounds—induce the expression of HSP27 leading to protection against apoptotic cell death.[6] HSP27 regulates apoptosis through interaction or modulation of key components of the apoptotic signalling pathways, including preventing the activation of caspases,[7] sequestering cytochrome c following its release from mitochondria[8] and stabilising F-actin which maintains compartment integrity and supports protein trafficking.[9–11] HSP27 also exerts an inhibitory effect on apoptosis by regulating the activation of Akt, a protein kinase that targets many apoptotic proteins.[12 13]

In this study we report the analysis of HSP27 in 719 patients with colorectal cancer in two independent cohorts. In the largest cohort analysed, high HSP27 expression was associated with poor outcome in rectal cancer. In the smaller cohort, which had approximately half the number of patients with rectal cancer and was characterised by earlier stage disease and significantly better overall survival, the relationship between HSP27 overexpression and poor outcome was not observed. The levels of HSP27 in preoperative diagnostic rectal biopsies were similar to those observed in matched surgical samples and were not affected by preoperative radiotherapy.

METHODS

Patients

Tissue microarrays from 404 formalin-fixed paraffin-embedded primary tumours (199 colon, 205 rectal) from consented patients who underwent surgery at the Royal Liverpool University Hospital, UK between 1993 and 2003 were obtained from the Cancer Tissue Bank Research Centre, University of Liverpool. One hundred and twenty-one patients (30%) underwent chemotherapy. In the 103 patients (85%) in whom the chemotherapy agents were known, all received 5-fluorouracil and four (4%) received an additional agent (irinotecan or oxaliplatin). Second-line and third-line chemotherapy was given in 37/103 (36%) and 19/103 (18%) patients, respectively. Additional agents (irinotecan, oxaliplatin, mitomycin C or levamisole) were administered in 9/37 (24%) second-round and 10/19 (53%) third-round patients. Thirteen per cent of patients with rectal cancer underwent short-course radiotherapy as part of the CR07 trial[14] (25 Grey administered in 5 fractions over 1 week with surgery within 1 week of treatment), while 15% underwent long-course radiotherapy (offered to patients with bulky tumours and consisting of 45 Grey administered in 25 daily fractions over 5 weeks followed by surgery after 6–10 weeks). Clinicopathological data including age, gender, site of tumour, stage (according to TNM and the American Joint Committee on Cancer guidelines[15]) are shown in table 1. The median follow-up was 49.5 months (IQR 19.6–74.6, range 0.23–146.5) and 284 deaths from any cause were reported, including 176 deaths due to colorectal cancer. Seven patients died within 30 days of surgery and were censored in the survival analysis. Four additional patients were censored at the point of last follow-up as no date of death was recorded.

Tissue preparation and two-dimensional electrophoresis

Twelve stage III cancers (9 colonic and 3 rectal) with varying survival times were submitted to two-dimensional electrophoresis with the aim of detecting proteins associated with prognosis. The surgical specimens were cryofixed in liquid isopentane and stored in liquid nitrogen. Tumour lysates were prepared by solubilising 30 × 8 μm undissected frozen sections in 400 μl lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-base). Protein quantities were normalised and two-dimensional SDS-PAGE was undertaken as described previously.[16] Gels were fixed and Coomassie Blue stained according to Neuhoff *et al.*[17] The gels were scanned using a GS-800 scanner (Bio-Rad, Hercules, Caliornia, USA) and images were acquired using PDQuest software (Bio-Rad V6.2). Image analysis was performed with Progenesis SameSpots imaging software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Identification of proteins by liquid chromatography tandem mass spectrometry (LC-MS/MS)

HSP27 was identified following gel spot excision and trypsin digestion according to Courchesne and Patterson.[18] The tryptic digest was delivered into a QSTAR Pulsar i hybrid mass spectrometer (AB Sciex, Warrington, UK) by automated in-line RP-LC (integrated LCPackings System, 5 mm C18 μ-precolumn cartridge and 75 μm × 15 cm C18 column, Dionex, California, USA) via a nano-electrospray source head and a 10 mm id PicoTip (New Objective, Massachusetts, USA) as previously described.[19] Data were submitted to MASCOT and the NCBI database was searched with the MS tolerance set to 1.2 Da and the MS/MS tolerance to 0.6 Da with carboxamidomethyl as a variable modification.

Tissue microarray construction, staining and scoring

Six purpose-built colorectal cancer tissue microarrays (TMAs) were constructed, containing 2–6 cores from 404 independent cases of adenocarcinoma in addition to 36 cores of normal colon and 16 cores each of normal kidney, liver and testes, which served as control tissues. Immunohistochemistry was undertaken as described previously[20] using mouse monoclonal anti-HSP27 (Novocastra, Newcastle-upon-Tyne, UK) diluted 1:50. TMAs were scored independently by two specialist histopathologists (authors BA and MT) who were blinded to all clinicopathological data. The intensity of staining on a scale of 0–3 and the extent of HSP27 staining on a scale of 0–3 according to the percentage of positive tumour (0, <5% positive cells; 1, 5–30%; 2, 30–70%; and 3, >70%) were recorded.

Statistical analysis

Comparisons were made using the non-parametric continuity corrected χ^2 test (2 groups) or χ^2 test (>2 groups). Life tables and Kaplan–Meier curves were used to evaluate the effect of HSP27 expression on cancer-specific survival; p values were calculated using the log rank test. Survival was measured from date of diagnosis to date of death, counting death from colorectal cancer as the end point; deaths from other causes were censored in the analysis. If no event occurred, the date of last follow-up was used as the end point. Multivariate Cox regression analysis was conducted using a stepwise forward selection approach starting with the most significant variable on univariate analysis and including every variable with a p value of <0.10.[21] All analyses were performed using Statview Version

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Table 1 Association between HSP27 expression and clinicopathological features

Clinical parameter	All cases				Colon cancers				Rectal cancers			
	Cases (N=404) n (%)	HSP27 −ve (N=228) n (%)	HSP27 +ve (N=176) n (%)	p Value*	Cases (N=199) n (%)	HSP27 −ve (N=115) n (%)	HSP27 +ve (N=84) n (%)	p Value*	Cases (N=205) n (%)	HSP27 −ve (N=113) n (%)	HSP27 +ve (N=92) n (%)	p Value*
Gender												
Female	153 (38)	83 (36)	70 (40)	0.489	95 (48)	51 (44)	44 (52)	0.262	58 (28)	32 (28)	26 (28)	0.993
Male	251 (62)	145 (64)	106 (60)		104 (52)	64 (56)	40 (48)		147 (72)	81 (72)	66 (72)	
Age												
<70 years	209 (52)	113 (50)	96 (55)	0.320	90 (45)	51 (44)	39 (46)	0.771	119 (58)	62 (55)	57 (62)	0.306
≥70 years	195 (48)	115 (50)	80 (45)		109 (55)	64 (56)	45 (54)		86 (42)	51 (45)	35 (38)	
Tumour size												
<40 mm	98 (24)	52 (23)	46 (26)	0.439	42 (21)	25 (22)	17 (20)	0.683	56 (27)	27 (24)	29 (31)	0.391
40–59 mm	178 (44)	98(43)	80 (46)		83 (42)	45 (39)	38 (45)		95 (46)	53 (47)	42 (46)	
≥60 mm	128 (32)	78 (34)	50 (28)		74 (37)	45 (39)	29 (35)		54 (27)	33 (29)	21 (23)	
Resection margins												
Clear	356 (88)	207 (91)	149 (84)	0.080	181 (91)	104 (90)	77 (91)	0.563	175 (85)	103 (91)	72 (78)	0.009
Involved	47 (12)	21 (9)	26 (15)		17 (9)	11 (10)	6 (8)		30 (15)	10 (9)	20 (22)	
Unrecorded	1	0	1 (1)		1	0	1 (1)		0	0	0	
Differentiation												
Well	10 (2)	4 (2)	6 (4)	0.562	4 (2)	1 (1)	3 (4)	0.087	6 (3)	3 (3)	3 (3)	0.316
Moderate	359 (89)	204 (89)	155 (88)		181 (91)	101 (88)	77 (91)		181 (88)	103 (91)	78 (85)	
Poor	31 (8)	18 (8)	13 (7)		15 (7)	12 (10)	3 (4)		16 (8)	6 (5)	10 (11)	
Unrecorded	4 (1)	2 (1)	2 (1)		2	1 (1)	1 (1)		2 (1)	1 (1)	1 (1)	
Depth of invasion												
T1	20 (5)	11 (5)	9 (5)	0.763	12 (6)	7 (6)	5 (6)	0.964	8 (4)	4 (3)	4 (4)	
T2	60 (15)	34 (15)	26 (15)		19 (9)	10 (9)	9 (11)		41 (20)	24 (21)	17 (19)	
T3	273 (67)	157 (69)	116 (66)		131 (66)	77 (67)	54 (64)		142 (69)	80 (71)	62 (67)	
T4	51 (13)	26 (11)	25 (14)		37 (19)	21 (18)	16 (19)		14 (7)	5 (4)	9 (10)	
Nodal status												
N0	225 (55)	131 (57)	94 (53)	0.558	117 (59)	65 (56)	52 (62)	0.744	108 (53)	66 (58)	42 (46)	0.095
N1	99 (25)	56 (25)	43 (25)		43 (22)	26 (23)	17 (20)		56 (27)	30 (27)	26 (28)	
N2	80 (20)	41 (18)	39 (22)		39 (19)	24 (21)	15 (18)		41 (20)	17 (15)	24 (26)	
Adjuvant therapy												
None	279 (69)	161 (71)	118 (67)	0.297	152 (76)	89 (77)	63 (75)	0.613	127 (62)	72 (64)	55 (60)	0.406
Chemotherapy	121 (30)	63 (27)	58 (33)		46 (23)	25 (22)	21 (25)		75 (37)	38 (33)	37 (40)	
Unrecorded	4 (1)	4 (2)	0		1 (1)	1 (1)	0		3 (1)	3 (3)	0	
Neoadjuvant therapy												
None	–	–	–		–	–	–		147 (72)	84 (74)	63 (68)	0.629
Short course	–	–	–		–	–	–		27 (13)	14 (12)	13 (14)	
Long course	–	–	–		–	–	–	–	31 (15)	15 (14)	16 (18)	

*Non-parametric continuity corrected chi-square test (2 groups) or chi-squared test (>2 groups).

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5.01 (SAS Institute Inc, Cary, North Carolina, USA). A p value of <0.05 was considered significant.

Assessment of HSP27 in an independent patient cohort

To determine whether the findings obtained using the patients from Liverpool, UK were also observed in an independent cohort of patients, TMAs containing 200 colon cancers and 115 rectal cancers resected consecutively from 1990 to 1995 in Düsseldorf, Germany[22 23] were stained for HSP27 expression and scored as described above; χ^2 testing was used to compare clinicopathological variable between the cohorts. HSP27 scores were compared between cohorts using the Mann–Whitney U test and correlation with cancer-specific survival was determined using Kaplan–Meier curves.

RESULTS

Proteomic analysis and identification of HSP27

Comparison of two-dimensional protein gels of 12 colorectal cancer specimens (figure 1A–C) revealed considerable variation in the intensities of the protein spot, indicated with an arrow (figure 1A, B). The spot was excised, subjected to in-gel trypsin digestion followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) which sequenced the peptide TKDGVVEITGK (figure 1D). This led to the identification of HSP27.

Correlation of parameters of HSP27 scoring with survival

To validate the variable HSP27 expression observed in colorectal cancer specimens, we undertook immunohistochemistry of TMAs (n=404 patients; Liverpool cohort). Normal colonic epithelium was devoid of HSP27 expression (figure 2A), whereas weak HSP27 immunostaining was observed in normal liver and intense staining was detected in normal renal tubules, as expected.[24 25] The variation in intensity of HSP27 expression in the colorectal cancer specimens was scored as follows: 0 (n=111), 1 (n=118), 2 (n=121), 3 (n=44) (figure 2B). The percentage of HSP27-positive tumour cells in the specimens was 0 (<10%), n=80; 1 (10–30%), n=99; 2 (30–70%), n=107; 3 (>70%), n=118 (figure 2C). A combined score or 0–9 index was calculated by multiplying the intensity by the extent of staining for every patient. For analysis, the intensity, extent and combined index were dichotomised by the median; intensity (low ≤1.5 vs high >1.5), extent (low ≤2 vs high >2) and combined index (low ≤3 vs high >3). These factors were subject to univariate Cox regression to assess which aspects of HSP27 staining were correlated with cancer-specific survival (see table 1 in online supplement). Neither the intensity (p=0.0955) nor the extent (p=0.7056) of staining was related to cancer-specific survival. However, the combined index (p=0.0320) was predictive of survival and was therefore the measure used in the construction of all subsequent Kaplan–Meier curves and life tables.

Clinicopathological data and correlation with HSP27 expression

Associations were sought between HSP27 expression (combined index) and gender, age, anatomical location, size of tumour, resection margin status, differentiation grade, depth of invasion

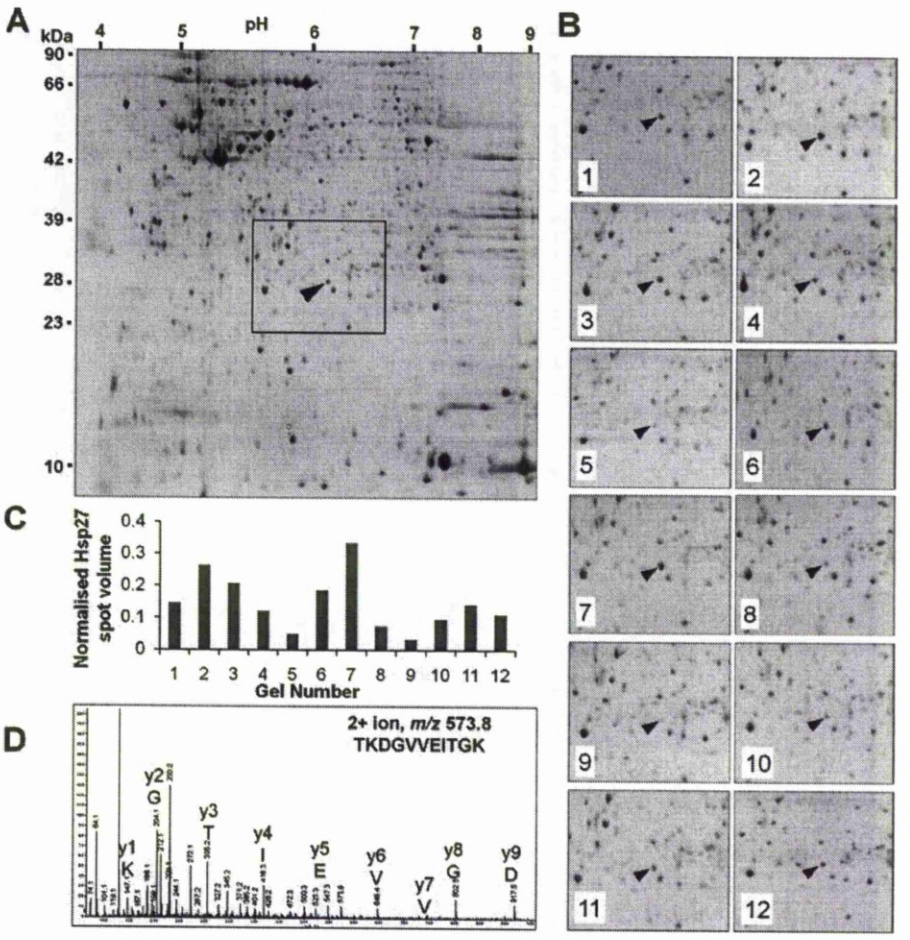

Figure 1 (A) Colloidal Coomassie Blue stained two-dimensional gel image of a colorectal cancer lysate with the HSP27-containing spot arrowed. (B) Insets from 12 independent colorectal tumour gels showing different intensities in the HSP27 protein spot. (C) Normalised HSP27 levels ((HSP27 spot intensity/(spot intensity, all spots)) less gel background). (D) MS/MS spectrum of peptides 113–123 from HSP27.

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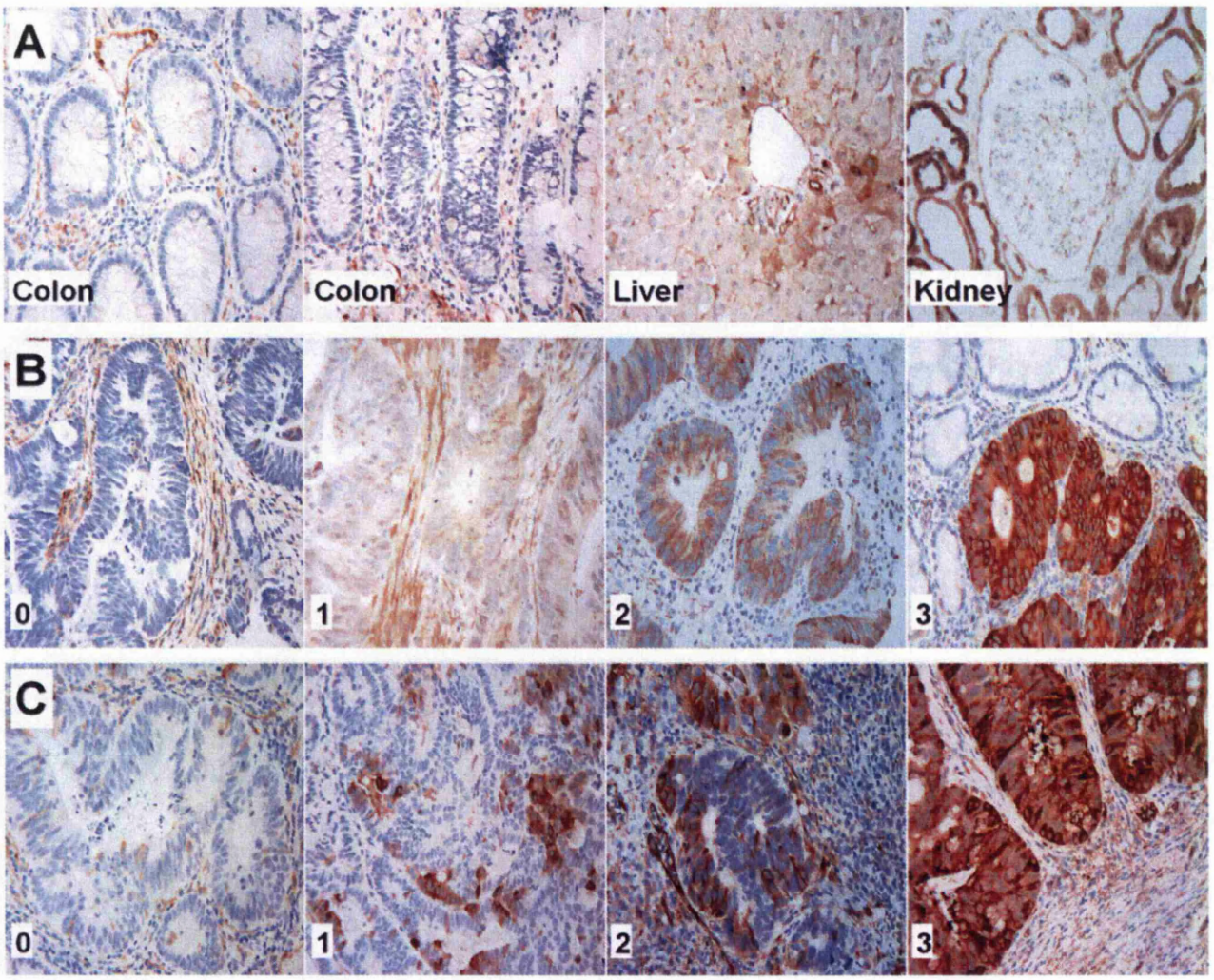

Figure 2 (A) Benign control tissue showing absence of detectable HSP27 in normal colonic epithelium, weak staining in normal liver and intense staining of normal renal tubules. (B) Colorectal cancer tissue illustrating the range of intensities of HSP27 immunostaining from 0 to 3. (C) Colorectal cancer tissue depicting varying percentages of HSP27 staining in different tumours.

(T stage), presence of nodal metastases (N stage) and treatment (adjuvant chemotherapy and neoadjuvant radiotherapy). The entire cohort of 404 patients was examined, in addition to separate analyses of the subgroups of patients with colon cancer (n=199) and rectal cancer (n=205). No significant correlations were found between the HSP27 index and these parameters in any of the groups analysed, except for an association between high HSP27 expression and incomplete resection margins in patients with rectal cancer (p=0.009; table 1).

HSP27 expression and association with survival

Elevated HSP27 was associated with poor survival (p=0.0312) when the entire Liverpool cohort of 404 patients was examined (figure 3A). However, when the patients with colon and rectal cancer were analysed separately, HSP27 expression was not associated with survival in the colon cancer group (p=0.7385; figure 3B), but was significantly associated with poor survival in the rectal cancer group (p=0.0063; figure 3C). At 5 years, rectal cancer-specific survival for high versus low HSP27 expressers was 63% vs 73%. Survival was assessed across all stages at presentation in rectal and colon cancer. Elevated HSP27 predicted poor cancer-specific survival in patients with rectal cancer with early-stage I/II tumours (figure 3D). However, HSP27 was not predictive of poor survival for patients with rectal cancer with stage III (N+/M0) disease (p=0.2132, figure 3E). No difference in survival according to HSP27 expression was noted in the colon cancer group (see figures 1A and B in online supplement).

The benefits of adjuvant therapy for patients with stage III disease are well established, while management of patients with stage II rectal tumours (T3–4/N0/M0) remains controversial.[26] We next determined whether high HSP27 expression might be useful in identifying a high-risk group of patients with stage II rectal tumours (T3–4/N0/M0) who might benefit from adjuvant therapy. We found that the overall survival of patients with stage II (T3–4/N0/M0) disease who had elevated HSP27 (n=29) was similar to that of patients with N1 disease (n=56, p=0.6523, figure 3F). This was not observed in colon cancer, where the overall survival of patients with stage II (T3–4/N0/M0) disease who had elevated HSP27 (n=41) was significantly better than that of patients with N1 disease (n=43, p=0.0268, see figure 1C in online supplement).

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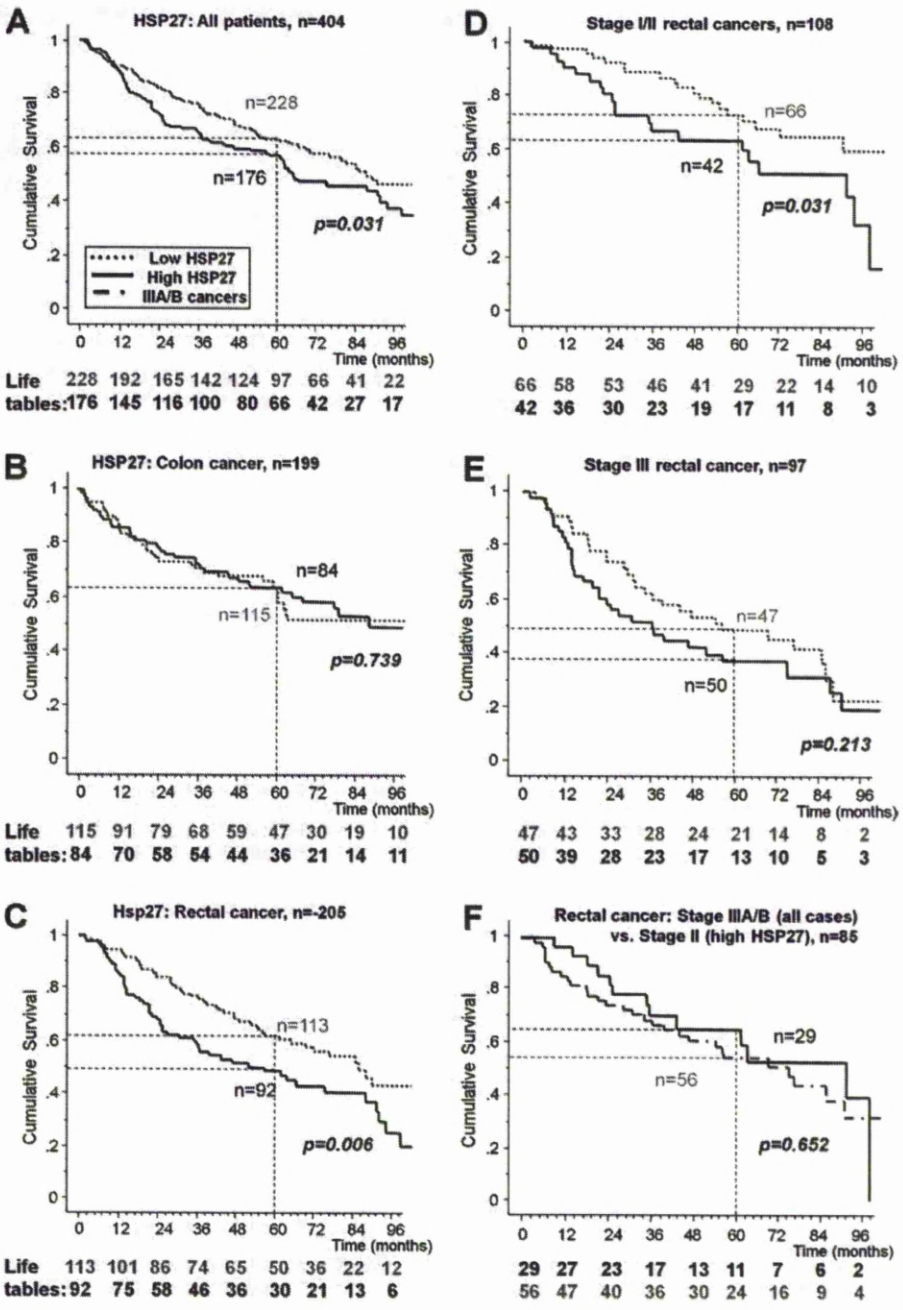

Figure 3 Kaplan–Meier graphs of 8-year survival in (A) all patients, (B) colon cancer patients, (C) rectal cancer patients, (D) stage I/II rectal cancer patients, (E) stage III rectal cancer patients and (F) stage IIIA/B rectal cancer patients versus stage II high HSP27 expressors.

Multivariate analysis

Multivariate Cox proportional hazards regression analysis was performed to derive risk estimates related to survival for all clinicopathological characteristics and HSP27 expression (table 2). Elements associated with cancer-specific survival on univariate analysis with $p<0.10$ were included in the multivariate model. Univariate analysis of 205 patients with rectal cancer showed that resection margin status (p=0.0894), N stage (p=0.0001) and HSP27 expression (p=0.0069) were significantly associated with survival. Only N stage and HSP27 expression remained independently predictive of survival with p values of 0.0001 and 0.0233, respectively (table 2). Univariate analysis of 199 patients with colon cancer showed significant factors to be differentiation grade (p=0.0278), T stage (p=0.0008), resection margin status (p=0.0001) and N stage (p=0.0001). HSP27 was not associated with survival (p=0.7382). On multivariate analysis, only resection margin status (p=0.0001) and N stage (p=0.0001) remained independently significant (see table 2 in online supplement).

Relationship between HSP27 expression and neoadjuvant treatment

The difference in survival observed in patients with rectal cancer but not those with colon cancer prompted us to question whether HSP27 expression was related to administration of neoadjuvant radiotherapy. Twenty-one patients with rectal cancer (13%) had short-course preoperative radiotherapy, 31 (15%) had long-course radiotherapy and 147 (72%) had no neoadjuvant therapy (table 1). Preoperative treatment did not

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Table 2 Cox proportional hazard regression model for rectal cancer (n=205)

Variables	Categories	Cases (N=205) n (%)	Univariate HR (95% CI)	χ^2	p Value	Multivariate HR (95% CI)	χ^2	p Value
Age (years)	68 (60–74)	–	1.000 (0.981 to 1.018)	0.001	0.9734	–	–	–
Size (mm)	45 (35–60)	–	1.001 (0.998 to 1.004)	0.115	0.6934	–	–	–
Differentiation grade	Well to moderate	187 (91)				–	–	–
	Poor	16 (9)	1.355 (0.682 to 2.693)	0.753	0.3857	–	–	–
T stage	T1	8 (4)	–	2.686	0.2611	–	–	–
	T2	41 (20)	1.154 (0.336 to 3.967)	0.052	0.8203	–	–	–
	T3	156 (76)	1.708 (0.539 to 5.416)	0.827	0.3630	–	–	–
Resection margins	Clear	175 (85)						
	Involved	30 (15)	1.563 (0.933 to 2.618)	2.884	0.0894	0.808 (0.450 to 1.453)	0.506	0.4769
N stage	N0	108 (53)	–	23.892	0.0001	–	20.227	0.0001
	N1	56 (27)	1.557 (0.962 to 2.521)	3.250	0.0714	1.539 (0.947 to 2.500)	3.026	0.0820
	N2	41 (20)	3.259 (2.027 to 5.240)	23.766	0.0001	3.289 (1.956 to 5.532)	20.150	0.0001
HSP27 expression	Negative	113 (55)						
	Positive	92 (45)	1.720 (1.160 to 2.550)	7.293	0.0069	1.607 (1.066 to 2.422)	5.143	0.0233

affect levels of HSP27 in the resected tumour (p=0.629, table 1). Diagnostic biopsy material was available for 80 of these patients and was stained and scored for HSP27 expression and compared with matched tissue from the TMAs (figure 4A; table 3 in online supplement). In 65/80 cases (81%), HSP27 expression was identical in the biopsy and the tumour. Five tumours (6%) showed upregulation of HSP27 and 10/80 (13%) showed downregulation of HSP27 in the surgical samples compared with the biopsy material (see table 3 in online supplement). Neoadjuvant therapy did not alter HSP27 expression between the biopsy and tumour specimens (p=0.6423, table 4 in online supplement).

Neoadjuvant therapy is not associated with cancer-specific survival

Having shown that neoadjuvant radiotherapy did not influence HSP27 expression, we sought to determine whether the type of

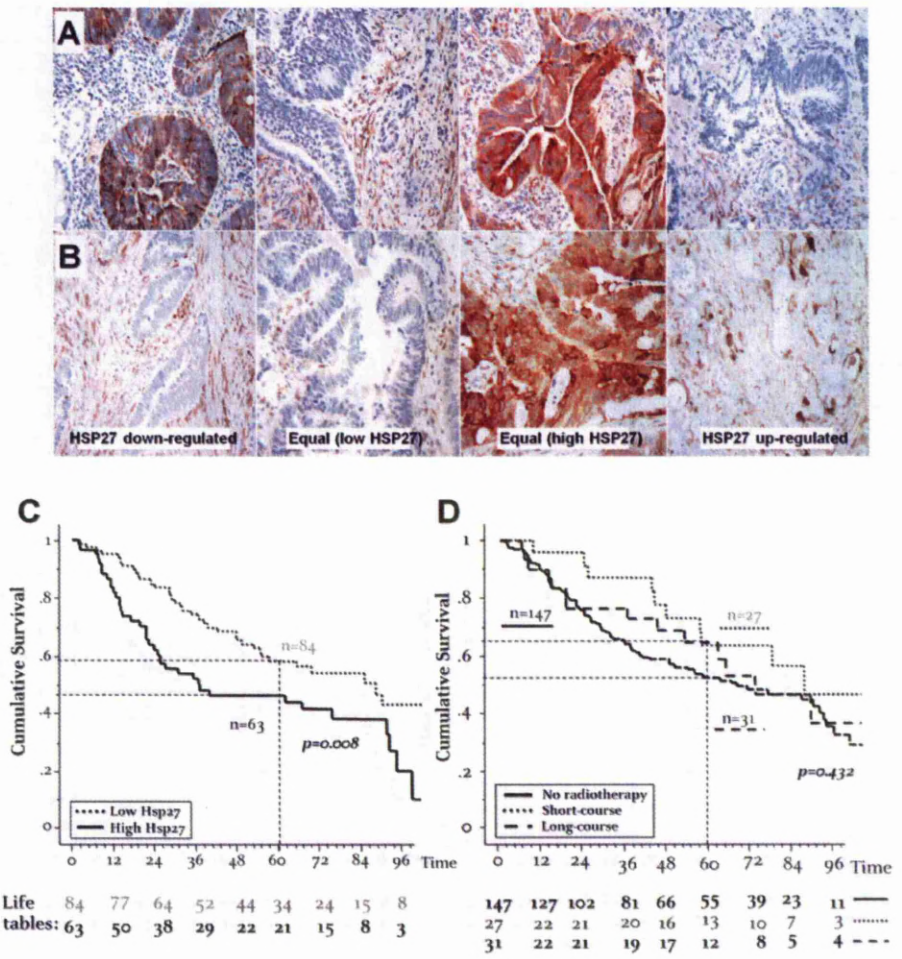

Figure 4 (A) HSP27 staining in diagnostic biopsy material compared with (B) matched tumour cores. (C) Kaplan–Meier survival curves showing that the level of HSP27 remains predictive of cancer-specific survival in patients with rectal cancer who have not received neoadjuvant radiotherapy and (D) that the mode of neoadjuvant radiotherapy received did not correlate with cancer-specific survival.

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neoadjuvant radiotherapy received contributed to the survival difference observed in the rectal cancer group. Survival analysis performed for the 147 patients with rectal cancer who did not receive radiotherapy treatment confirmed that high HSP27 expression in this group was associated with poor survival (p=0.0078, figure 4B). No difference in cancer-specific survival was seen in the rectal cancer group according to type of radiotherapy received (p=0.4317, figure 4C).

Analysis of HSP27 expression in an independent cohort of patients

Finally, to examine the relationship between HSP27 expression and outcome in an independent cohort of patients with colorectal cancer, analysis of HSP27 expression was undertaken for 315 patients with colorectal cancer resected consecutively between 1990 and 1995 in Düsseldorf, Germany. We observed a lower median proportion of tumour staining for HSP27 in the Düsseldorf cohort (1.25 vs 2.0, p<0.0001; see table 3 in online supplement), although intensity of staining and HSP27 index showed no significant difference (p=0.064 and p=0.207, respectively; table 3 in online supplement). The Düsseldorf cohort was dichotomised by the median HSP27 index score into ≤2.5 (n=159) or >2.5 (n=156), as was the case for the Liverpool cohort. No significant difference in survival was observed by HSP27 expression in the Düsseldorf group overall (p=0.327; figure 2A in online supplement) and, consistent with the Liverpool cohort, HSP27 expression did not correlate with survival of patients with colon cancer (n=200) in the Düsseldorf cohort (p=0.713; figure 2B in online supplement). However, in contrast to the finding that HSP27 overexpression correlated with poor outcome in the Liverpool cohort, HSP27 overexpression was not significantly associated with survival in patients with rectal cancer (n=115) in the Düsseldorf cohort (p=0.308; figure 2C in online supplement).

In order to determine why high HSP27 expression predicted a poor outcome in the Liverpool cohort but not in the Düsseldorf cohort, the clinicopathological characteristics and cancer-specific survival of the two cohorts were compared (see table 4 in online supplement). The overall 5-year survival of the Düsseldorf cohort was 69%, which was significantly better than the Liverpool cohort (59%; p=0.0002). The patients with colon cancer had similar distributions of age (p=0.481), gender (p=0.089), T and N staging (p=0.079 and 0.139, respectively) in the respective cohorts. Nonetheless, cancer-specific survival for patients with colon cancer was superior in the Düsseldorf group (69% vs 65% at 5 years, p=0.02) and fewer patients with colon cancer had adjuvant therapy in the Düsseldorf group than in the Liverpool group (p=0.001). With respect to rectal cancer, the cohorts were considerably different with fewer men (p=0.0001) and fewer tumours with advanced stages (T3/4 or N2 cases; p=0.007 and p=0.044, respectively) in the Düsseldorf group. Conversely, Liverpool patients were more likely to have well/moderately differentiated tumours (p=0.0004) and to have had adjuvant therapy (p≤0.0001). Patients with rectal cancer showed the greatest difference in cancer-specific 5-year survival (69% for the Düsseldorf cohort vs 56% Liverpool cohort, p=0.007).

DISCUSSION

Clinical staging plays a significant role in predicting survival and guiding treatment for patients with rectal cancer.[27] However, it is acknowledged that patients at similar clinical stages can have very different outcomes, and biomarkers capable of identifying patients at high risk who might benefit from adjuvant chemotherapy are particularly required. Carcinoembryonic antigen (CEA) is the only biomarker recommended by the American Society of Clinical Oncology and the European Group on Tumour Markers for use in the postoperative period for the early detection of recurrent or metastatic rectal cancer. There is evidence that high preoperative CEA levels are independently associated with poor survival, but the data are insufficient to support the use of CEA to determine treatment with adjuvant therapy. There are currently no data to support the use of thymidine synthase (TS), p53 mutation, k-ras mutation, DNA ploidy or 18q deletion for determining prognosis or response to treatment in rectal cancer.[28 29] Preoperative chemoradiotherapy is recommended for the treatment of locally advanced rectal cancer following the results of a number of randomised controlled trials.[30 31] DNA analysis of tumour tissue has revealed that genes associated with DNA repair, apoptosis and cell adhesion are related to radioresistance.[32] However, there are no reliable markers which predict response to neoadjuvant therapy in individual patients prior to treatment.[33]

Here we report that, in a cohort of 205 patients with rectal cancer from Liverpool, high HSP27 expression is an independent marker of poor outcome. In the smaller Düsseldorf cohort of patients with rectal cancer (n=115), the relationship between HSP27 and survival in rectal cancer was not observed. This may relate to the fact that the Düsseldorf cohort had significantly fewer men (a poor prognostic group), a greater proportion of early (N0) cancers and at 7% better 5-year cancer-specific survival. The latest Eurocare study confirms a discrepancy in relative 5-year survival for patients with colorectal cancer between the UK and Germany (51.8% vs 61.2%),[34] which is consistent with the difference we have observed between the two cohorts in this study. We postulate that the significantly higher proportion of early rectal tumours in the Düsseldorf group and the correspondingly better outcomes in these patients reduced our ability to detect a significant survival difference according to HSP27 expression in this small group of patients.

Increased HSP27 in colorectal tumour specimens has been reported in smaller studies[35 36] with inconclusive findings. Pei *et al* reported a strong relationship between HSP27 expression in the primary tumour and nodal status (n=40),[35] while Zhao *et al*[36] found no such association (n=68). Our data represent the largest clinical analyses of HSP27 expression in colorectal adenocarcinoma, and the first study in which HSP27 levels were analysed in colon and rectal cancer separately. Our finding that HSP27 expression has prognostic value in rectal but not colon cancer adds to accumulating evidence that rectal and colon tumours are distinct biological entities.[37]

Given the role of HSP27 in apoptosis, it is not surprising that its levels in many cancer types have been examined and relationships to outcome determined.[38] For cancers such as prostate,[39 40] ovarian,[41] gastric[42] and hepatocellular carcinoma,[24] high levels of HSP27 are associated with a poor prognosis. In contrast, HSP27 expression has been associated with a good prognosis in endometrial,[43] oesophageal[44] and oral squamous cell carcinoma.[45] Findings remain inconclusive in breast cancer, with high HSP27 linked to poor prognosis in some studies[46 47] but no association being detected in others.[48 49] Using raised HSP27 as a biomarker, we defined a poor prognostic group of stage II patients in the Liverpool cohort with survival similar to that of stage IIIA/B patients. Further work will be required to establish whether this group of patients would benefit from chemotherapy. HSP27 expression was independently associated with survival in patients with rectal cancer on multivariate analysis. We were interested to note that resection margin status

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was not independently significant in the multivariate analysis, which was contrary to our expectations. This may reflect non-standardised pathological data collection prior to 1999 in Liverpool, when total mesorectal excision and the minimum pathological dataset were introduced.

Our analysis of HSP27 expression in preoperative biopsy material from a subset of the patients with rectal cancer afforded us the opportunity to make two important observations. First, the expression of HSP27 levels in the tumours was stable over time, with 81% showing no change in expression between biopsy and surgical sample. The mechanism by which HSP27 is induced in cancer is not fully understood, although features of the tumour microenvironment as well as overproduction and misfolding of mutated proteins in cancer cells have been suggested as inducing factors.[50] Whatever the cause of induced HSP27 expression in rectal cancers, our data indicate a sustained overproduction of the protein. Second, we observed that radiotherapy does not alter the expression of HSP27 between diagnosis of rectal tumours and surgical resection. Our findings concur with the only published study of HSP27 expression in relation to radiotherapy treatment of rectal cancer, which found no consistent change in HSP27 expression after radiotherapy was administered (n=23).[51] The low proportion of patients undergoing neoadjuvant therapy in our cohort reflects the practice at the time of diagnosis (1993–2003) when preoperative radiotherapy in rectal cancer was not standard.

A variety of mechanisms by which HSP27 exerts its effects on survival in cancer have been proposed, including enhanced motility and invasion,[52 53] inhibition of apoptosis[6–8] and resistance to anti-cancer treatments.[6] The role of HSP27 in chemoresistance has been intensely studied and the protein has been implicated in 5-fluorouracil and irinotecan resistance in colorectal cancer.[54 55] Of particular interest in rectal cancer is the role that HSP27 plays in resistance to radiotherapy. There is in vitro evidence that HSP27 confers resistance to gamma-radiation in human cancer cell lines.[56] Further work is required to establish whether a link exists between HSP27 expression, resistance to radiotherapy treatment and prognosis in rectal adenocarcinoma.

In conclusion, elevated HSP27 is an independent marker of poor prognosis in rectal cancer. Expression of the protein appears stable between the time of biopsy and tumour specimen acquisition and it is not altered by neoadjuvant radiotherapy. Further work is necessary to validate these findings in a larger group of patients with rectal cancer and to determine whether HSP27 has a role to play in resistance to adjuvant radiotherapy and chemotherapy in vivo.

Funding This work was supported by a Royal College of Surgeons of England Fellowship to EMT and by joint CR-UK/NIHR funding grant number ECMC GRANT C6665/A7348.

Competing interests None.

Ethics approval This study was conducted with the approval of the Liverpool LREC.

Provenance and peer review Not commissioned; externally peer reviewed.

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